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ENDOCRINOLOGY

VOLUME 36 JANUARY, 1945 NUMBER 1

THE ABSORPTION OF VARIOUS GONADOTROPIN TANNATES IN THE BODY[1,2]

FELIX SULMAN, SH. LEVY-HOCHMAN AND R. BLACK

From the Hormone Research Laboratory of the Hebrew University and the Obstetrical-Gynecological Department of the Rothschild Hadassah University Hospital

JERUSALEM, PALESTINE

EXPERIMENTS by Maxwell (1934) and Bischoff (1934) showed that gonadotropins combined with tannin tend to be absorbed relatively slowly. The induced delay in absorption produced remarkable augmentation of the effect of pituitary gonadotropins, but not of pregnant mare serum gonadotropin or of human chorionic gonadotropin.

We have undertaken a series of experiments to study the absorption of various gonadotropin-tannin-preparations.

MATERIALS AND METHODS

The following hormones were studied:

(a) Chorionic gonadotropin. This was prepared from urine of pregnant women either according to the alcohol method of B. Zondek (1928, 1931, 1935a) or by the tannic acid method of Levin & Tyndale (1936). Since there is ample proof that both methods precipitate the gonadotropin quantitatively, we were able to compare powders prepared by these methods from the same sample of urine, provided that aliquot amounts of the powders were used. In the alcohol method, the urine is poured into 4 volumes of 96 per cent alcohol; in the tannic acid method, freshly prepared 10 per cent aqueous solution of tannic acid is added to the urine at PH 4.7 in amounts sufficient to produce maximal precipitation. The precipitates were allowed to settle over night in the refrigerator, collected by centrifugation on the following day and, after being washed twice with 96 per cent alcohol, acetone and ether, were dried in evacuation desiccators. The powders were then weighed and aliquots determined according to the yield.

(b) Pregnant mare serum gonadotropin. This was prepared from an ace-

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[1] Aided by a grant from the Ella Sachs Plotz Foundation.

[2] Because of the difficulties of international communication the authors have not read proof of this article.

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tone dried powder of pregnant mare serum which was dissolved in water and then treated with alcohol or tannic acid as described above.

(c) Bovine pituitary gonadotropin. This was extracted according to the method of Van Dyke and Wallen-Lawrence (1933) from an acetone dry powder of ox anterior lobes with ammonia-water and alcohol and then was treated as described under (a).

Titers of gonadotropins prepared as mentioned in (a) and (b) were determined originally in international units. Since we were working with rats, it was necessary to standardize the gonadotropins under identical conditions on infantile female rats. The rat unit (R.U.) as used in this paper is the follicle ripening unit defined by Zondek (1926) and is based on a full estrus vaginal smear of at least 60 per cent of 5 immature female rats used for any assay. This follicle maturation unit is much smaller than the international unit (I.U.). In our rats the relation of the two was as follows:

chorionic gonadotropin	1 I.U. = 5 R.U.
pregnant mare serum gonadotropin	1 I.U. = 10 R.U.

Comparison of the duration of the gonadotropic effect of the soluble gonadotropins and the insoluble tannin-gonadotropins was carried out with equal amounts of rat units.

In some of the experiments the hormone pellets were implanted intramuscularly into the gluteal muscles of infantile female rats. In this method, one factor was not subjected to rigid control, namely the pressure used for the preparation of the pellets. As a rule, the pellets were prepared by the same worker using maximal pressure of the right hand. The uniformity of the results reported indicates that the inaccuracy thus introduced is insignificant. Experiments were carried out on a total of 9 adult male rabbits and 223 infantile female rats.

EXCRETION OF CHORIONIC GONADOTROPIN ADMINISTERED IN FREE FORM AND AS TANNATE

Chorionic gonadotropin injected into laboratory animals is rapidly excreted in the urine, the total recovery of hormone from the urine amounting, however, only to 5 per cent of the dose given (Zondek, 1935b, 1941). Our findings suggest a main excretion period in rats of about 24 hours (Zondek, Sulman and Sklow, 1940, Zondek and Sulman, 1942). The same holds also for the rabbit (Salmon and Hamblen, 1943). Further data on this point are listed in the papers of Parkes and White (1933), Stamler (1937), Friedman and Weinstein (1937).

Three male rabbits, weighing appr. 1250 gm. each, received a dose of 600 I.U. (=3000 R.U.) of chorionic gonadotropin by various routes of application. One rabbit received an intramuscular injection of chorionic gonadotropin in aqueous solution, a second received an intramuscular injection of chorionic gonadotropin tannate in aqueous suspension, and a third 4 pellets of chorionic gonadotropin tannate (total weight 200 mg.) by intramuscular implantation. The 3 rabbits were kept in metabolism cages under similar conditions. The urines were collected at intervals of 24 hours. All samples were concentrated

to $\frac{1}{5}$ their original volume by the alcohol method. The gonadotropin content was assayed on infantile female rats (table I).

TABLE I. EXCRETION OF VARIOUS PREPARATIONS OF CHORIONIC GONADOTROPINS IN THE URINE OF RABBITS

Animal	Weight	Preparation	Administration	Amount given	Bulk of Excretion	Recovery
				I.U.	hour	%
Rabbit male	1250 g.	Alcohol method solution	intramuscular injection	600	24	5
Rabbit male	1250 g.	Tannic acid method suspension	intramuscular injection	600	120	5
Rabbit male	1250 g.	Tannic acid method pellets	intramuscular implantation	600	144	5

In 3 series of experiments, about 5 per cent of the amount of chorionic gonadotropin given was recovered from the urine in all three cases. There were striking differences, however, in regard to the day of excretion of this amount. Rabbits which received an injection of chorionic gonadotropin in aqueous solution excreted the hormone on the *1st day* after the injection. Rabbits given hormone in the form of an injection of an aqueous suspension of tannate excreted most hormone on the *5th day* after the injection. Rabbits implanted with 4 pellets of the tannate, excreted the major part of the hormone only *6 days* after they were implanted. At other times only subthreshold doses were excreted.

These experiments prove that the administration of chorionic gonadotropin as the tannate delays absorption for 5–6 days in comparison to gonadotropin in aqueous solution. Implanted pellets of gonadotropin tannate are absorbed slightly more slowly than injected tannate in suspension. These findings were corroborated in our further experiments.

ABSORPTION OF GONADOTROPINS ADMINISTERED BY INJECTION AND BY IMPLANTATION

200 infantile rats were implanted with pellets of various gonadotropins (human chorionic gonadotropin, pregnant mare serum gonadotropin, and bovine pituitary gonadotropin) in the gluteal muscles (table II). Three groups (Nos. 1, 3, 5) were implanted with gonadotropins prepared by alcohol precipitation, and five others (Nos. 2, 4, 6, 7, 8) with gonadotropins prepared by tannic acid precipitation. The duration of the activity of the pellets was tested by reimplanting them successively into infantile rats. When a gonadotropic effect was induced by a reimplanted pellet, it was considered to be still gonadotropically active. The pellets contained 10 or 20 I.U. The result is obvious from Table II. Tannate precipitate of all three types of gonadotropins tested was absorbed more slowly than was the corre-

sponding alcohol precipitate. The slowing down of absorption amounted to from 4 to 6.5 days.

TABLE II. DURATION OF THE ACTIVITY OF VARIOUS GONADOTROPIN PELLETS PREPARED BY ALCOHOL OR TANNIC ACID PREPARATION

Assay No.	Rats tested	Hormone preparation	Units	Days of activity Average
1	30	Chorionic Gonadotropin alcohol precipitate	20 I.U. = 100 R.U.	1(±1)
2	50	Chorionic Gonadotropin tannic acid precipitate	20 I.U. = 100 R.U.	7.5(±2.5)
3	15	Pregnant Mare serum Gonadotropin alcohol precipitate	10 I.U. = 100 R.U.	1
4	15	Pregnant Mare Serum Gonadotropin tannic acid precipitate	10 I.U. = 100 R.U.	6(±1)
5	25	Pituitary Gonadotropin alcohol precipitate	100 R.U. or 50 mg. resp.	2
6	35	Pituitary Gonadotropin tannic acid precipitate[1]	100 R.U. or 50 mg. respectively	6
7	20	Chorionic Gonadotropin tannic acid precipitate containing 50% cholesterol	20 I.U. = 100 R.U.	5(±1)
8	10	Pituitary Gonadotropin tannic acid precipitate containing 50% cholesterol	20 I.U. = 100 R.U.	4

[1] With this type of hormone a certain augmentative effect was observed. The ovary weights of the rats implanted with pituitary gonadotropin tannate averaged 100 per cent higher than did those of rats implanted with the alcohol precipitate.

In the experiments with pituitary gonadotropin a marked Maxwell effect was encountered, whereas with chorionic and pregnant mare serum gonadotropins, the gonadotropic response was never enhanced by the tannin treatment. The treatment with tannic acid increased the titer of pituitary gonadotropin about fourfold. The two forms of pituitary gonadotropin were therefore compared in one experiment by implanting pellets of equal gonadotropic activity (100 R.U.), and in a second by implanting pellets of equal weight (50 mg.). The difference in result was, however, insignificant.

While this work was in progress, there appeared a paper by Parkes (1942) describing the effect of chorionic gonadotropins administered in cholesterol pellets. Using pellets containing 200 I.U. chorionic gonadotropin in 90 per cent cholesterol Parkes found the gonadotropic effect to persist for a maximum of 6 days. In view of this result we examined the effect of chorionic gonadotropin tannate and pituitary gonadotropin tannate in pellets containing 50 per cent cholesterol. As the results compiled in Table II (Nos. 7 and 8) show the absorption of both gonadotropins was somewhat quicker from pellets containing cholesterol, than from pellets without cholesterol. Pellets containing cholesterol are necessarily greater in bulk and not homogeneous. It is possible that the increase in surface together with the loss of homo-

geneity are responsible for the quicker absorption of the cholesterol pellets.

THE GONADOTROPIC EFFECT OF VARIOUS GONADOTROPINS ADMINISTERED ORALLY

The stability of gonadotropin tannates when administered parenterally suggested the advisability of experiments to test the peroral effect of such preparations. Zondek (1935c) noted marked variations in the gonadotropic effect induced by chorionic gonadotropin given orally. Small oral doses did not in rats and mice yield a positive result (1931), whereas larger doses of chorionic gonadotropin taken orally by rats with milk (1935c) elicited a gonadotropic reaction. Zondek found that 2–3 I.U. (=10–15 R.U.) were sometimes sufficient to evoke follicle maturation, and that larger amounts such as 20 I.U. (=100 R.U.) gave surer results. To evoke luteinization 100–140 I.U. (=500–700 R.U.) were necessary. Further data have been given by Reiss and Haurowitz (1929), Dickens (1930), Janssen and Loeser (1931), Huddleston and Whitehead (1931).

We have treated 13 infantile female rats with 4–400 I.U. (=20–2000 R.U.) of chorionic gonadotropin tannate administered as a suspension in 1.5 cc. water by stomach tube. Positive gonadotropic reactions were observed in two cases only, the one showing follicle maturation, and the second maturation and luteinization. Both reactions were obtained following the administration of 50 I.U. (=250 R.U.). Even doses of 400 I.U. (=2000 R.U.) of chorionic gonadotropin tannate, however, did not ensure positive results. In 10 infantile female rats treated with 4–400 I.U. (=20–2000 R.U.) of soluble chorionic gonadotropin we observed only one case of follicle maturation, this following an administration of 300 I.U. (=1500 R.U.).

Our results confirm the earlier reports and exemplify strikingly the uncertainty of the gonadotropic response to chorionic gonadotropin when it is given orally. Oral administration of chorionic gonadotropin in the form of tannate does not lessen this uncertainty.

SUMMARY

1) The absorption of various insoluble gonadotropin tannates was studied in rats and rabbits and compared with that of soluble gonadotropins.

2) The urinary excretion of chorionic gonadotropin in rabbits which had received 600 I.U. (=3000 R.U.) of the gonadotropin in tannate form by different routes has been studied. In all cases approximately 5 per cent of the amount administered was excreted. The bulk of excretion occurred in the following intervals after administration:

Native gonadotropin solution, injected intramuscularly	1st day
Gonadotropin tannate suspension, injected intramuscularly	5th day
Gonadotropin tannate pellet, implanted intramuscularly	6th day

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3) The average duration of effectivity of pellets containing 100 R.U.[3] of various gonadotropins implanted intramuscularly into infantile female rats was the following:

Gonadotropin pellet preparation	Chorionic gonadotropin	Pregnant mare serum gonadotropin	Ox pituitary gonadotropin
Soluble after alcohol precipitation	1 day	1 day	2 days
Insoluble tannate preparation with 50 per cent cholesterol	5 days	not studied	4 days
Insoluble tannate preparation alone	7.5 days	6 days	6 days

4) Tannin enhanced the gonadotropic effect (Maxwell phenomenon) of implanted pellets of pituitary gonadotropin, but not of pellets of chorionic gonadotropin or pregnant mare serum gonadotropin.

5) When chorionic gonadotropin, whether in free form or as the tannate, was administered to infantile female rats orally, its effect was irregular.

ACKNOWLEDGMENT

We are indebted to Prof. Bernhard Zondek, head of the Department, under whose supervision this work was carried out.

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[3] The use of R.U. instead of I.U. has been explained in section (1).

METABOLIC CHANGES IN ESTROGEN INDUCED BY RAT ORGANS[1]

JOSEPH SCHILLER

From the Physiological Laboratory, Boston University School of Medicine and the Physiological Laboratories, Clark University

WORCESTER, MASS.

IN A PRELIMINARY PAPER (Schiller and Pincus 1943b) we presented data demonstrating that estradiol perfused through rat livers is converted to a ketonic estrogen (probably estrone) and to a "strong" phenolic estrogen (probably estriol). In this paper we present in detail the results of the perfusion of rat livers, kidneys, and heart tissue by various estrogens.

MATERIALS AND METHODS

The experimental animals employed were 225 to 250 gram female rats of the Sherman strain which were sacrificed under light ether anesthesia during proestrus. Standard aseptic conditions were maintained in preparing the organs for perfusion. Thorough and rapid bleeding of each donor was accomplished by exsanguination from the aorta. The liver *in situ* was, after bleeding, rapidly washed with Ringer-Locke solution, dissected and cannulated through the portal vein.

The kidneys were obtained from animals which were eviscerated. All the abdominal branches of the aorta, and the branch of the renal artery leading to the adrenals were ligatured. The animal was then bled through a cut made well below the renal artery. When bleeding ceased the aorta was ligated at the cut, and the kidneys washed with Ringer-Locke through a second cut made in the aorta well above the renal artery. The entire preparation, containing both kidneys and a length of aorta was dissected out, placed in a sterile petri dish and cannulated through the second cut in the aorta. The rat heart was perfused by cannulation through the aorta, again after rapid bleeding and washing with Ringer-Locke solution.

In all operations the time from the beginning to the mounting of the cannulated specimen in the perfusion apparatus never exceeded ten minutes, and was usually much less. The pyrex glass cannulae employed have a ground glass joint for connection to a ground male joint at the end of the inlet to the organ chamber of the perfusion pump. The glass perfusion apparatus was kept in an incubator at 37°C.

Extraction of estrogens from the perfusate is accomplished by: (a) addition of an alcohol: ether mixture (3 parts 95% ethyl alcohol to one part

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ethyl ether) using 18 cc. for each cc. of blood or serum in the medium; (b washing the ensuing precipitate with 95% ethyl alcohol using one-fourth to one-half the volume of the original alcohol: ether mixture; (c) combining the clear filtrates of (a) and (b), evaporating off the solvent and extracting the watery residue thoroughly with ethyl ether; (d) extracting the ether extract with 1 N NaOH which is then acidified (acid to congo red), and reextracted with ethyl ether. The phenolic fraction so obtained is evaporated to dryness. An aliquot is usually taken for estrogen bioassay by a modified Allen-Doisy method (Pincus and Pearlman 1941). Separation of the balance of the phenolic fraction into three fractions representing principally estradiol, estrone, and estriol is accomplished by methods previously described (Pearlman and Pincus 1943). Hydrolysis of portions of the perfusate when performed is accomplished by autoclaving at 14 lbs. pressure for 20 minutes after bringing the medium to pH 1–2 with HCl.

The extraction of liver is accomplished by adding to each liver 15 cc. of distilled water and 90 cc. of acetone (c.P.). The mixture is added to a Waring blender and brought to a soupy consistency. After centrifuging, the supernatant, containing the free estrogens, is dried and taken up in 140 cc. of petroleum ether. The petroleum ether solution is shaken twice with 35 cc. of 0.1 N NaOH to remove the estrogens (Mather 1942). The NaOH extracts are combined, neutralized with HCl and extracted thoroughly with ethyl ether. Fractionation of the free estrogens in the ethyl ether is accomplished as indicated previously. To obtain the bound estrogens the precipitate from the aqueous acetone is taken up in 9% NaCl and brought to pH 1–2 with HCl. It is hydrolized in the autoclave for twenty minutes at 14 lbs. pressure, and subsequent estrogen extraction carried on as indicated above.

The estrogens were added to the perfusing medium by dissolving them in 0.5 N NaOH with one drop of ethyl alcohol, bringing the solution carefully to pH 7.4 with HCl and adding this solution carefully to the perfusate. The estrogens remained in solution.

RESULTS

Rabbit serum extracted by our methods proved to have no detectable estrogenic activity (less than 1 R.U. in 18 cc.). On adding 300 R.U. of α-estradiol to rabbit serum and incubating for 3 and 17 hours 300 R.U. were recovered in each instance. Zondek (1934) has adequately demonstrated that rat blood exerts no detectable effect on added estrogen, so we contented ourselves with the circulation of 3200 R.U. of α-estradiol in 20 cc. defibrinated blood plus 100 cc. Ringer-Locke solution for one hour through the perfusion pump. 3000 R.U. were recovered on extraction, the 200 R.U. lost probably representing losses in manipulation though the value recorded lies within the error of our assay method.

In table 1 are the data on estrogen recoveries after perfusion of rat heart, liver, and kidney.

The data on α-estradiol perfusion demonstrate the following: After perfusion of the rat heart for three hours practically all of the activity is recovered and is present in the "estradiol" fraction (experi-

ment 1), the small amount in the "estrone" fraction being due in all likelihood to fractionation error. Perfusion of the liver with the same amount of α-estradiol (300 R.U.) for the same time results in a loss of

TABLE I. THE RECOVERY OF ESTROGENIC ACTIVITY FROM THE PERFUSING MEDIUM IN VARIOUS PERFUSIONS OF RAT ORGANS *in vitro*

Exp. No.	Perfusion Medium	Estrogen in Perfusate (r.u)	Perfusion time hrs.	Activity recovered from perfusate (r.u.)[1]			
				Total	Non-ketonic "estradiol"	Ketonic "estrone"	Na_2CO_3 fraction "estriol"
			Heart				
1	150 cc. rabbit serum 75 cc. Ringer-Locke	α-estradiol 300	3	270	270	4	0
			Liver				
2	25 cc. defibrinated rat blood 100 cc. Ringer-Locke	α-estradiol 3200	3	1000	400	400	200
3	20 cc. defibrinated blood 100 cc. Ringer-Locke	α-estradiol 3200	6	570[2]	220	220	132
4	25 cc. defibrinated blood 100 cc. Ringer-Locke	α-estradiol 4080	3[5]	1200[3] 1000[4]	—	—	—
5	80 cc. rabbit serum 15 cc. Ringer-Locke	α-estradiol 1664	6	134[3] 128[4]	—	—	—
6	25 cc. defibrinated blood 100 cc. Ringer-Locke	α-estradiol 300	3	43	—	—	—
7	20 cc. defibrinated blood 100 cc. Ringer-Locke	α-estradiol 208	5	15	—	—	—
8	25 cc. defibrinated blood 100 cc. Ringer-Locke	0	3	14[6]	—	—	—
9	75 cc. rabbit serum 50 cc. Ringer-Locke	estrone 3000	3	2520	936	798	663
10	75 cc. rabbit serum 50 cc. Ringer-Locke	estrone 3000	6	1410[7]	663	400	200
11	125 cc. rabbit serum 10 cc. Ringer-Locke	estrone 3000	10	—	327	252	76
12	75 cc. rabbit serum 50 cc. Ringer-Locke	estriol 2000	3	1400[8]	less than 10	R.U. in benzene	1400
			Kidney				
13	75 cc. rabbit serum 50 cc. Ringer-Locke	α-estradiol 4160	3	1860	876	711	41
14	75 cc. rabbit serum 50 cc. Ringer-Locke	α-estradiol 4160	6	1425[9]	615	500	70
15	75 cc. rabbit serum 50 cc. Ringer-Locke	α-estradiol 4160	10	124[10]	50	28	12

[1] By our assay method 1 R.U. α-estradiol =0.125 microgram
1 R.U. estrone =1.0 microgram
1 R.U. estriol =1.0 microgram
[2] An aliquot taken at 3 hrs. assayed 1000 R.U.
[3] Regular extraction.
[4] After acid hydrolysis.
[5] ½ of total plus 416 R.U. α-estradiol assayed 700 R.U., ½ of total acid hydrolized plus 416 R.U. α-estradiol assayed 730 R.U.
[6] Liver weight 6.6 gm.
[7] Aliquot taken at 3 hrs. assayed 2500 R.U.
[8] An aliquot hydrolized assayed 1400 R.U.
[9] An aliquot taken at 3 hrs. assayed 2000 R.U.
[10] An aliquot taken at 6 hrs. assayed 1500 R.U

85% of the activity (experiment 6). The loss of activity on liver perfusion for three hours with larger amounts of α-estradiol is somewhat less than with smaller amounts being 69% when 3200 R.U. were used (experiment 2 and 3), and 71% when 4080 R.U. were used (experiment 4). Perfusion of the liver for six hours leads to a further loss of activ-

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ity from the perfusate amounting to 82% when 3200 R.U. were used (experiment 3) and 92% when 1664 R.U. were used (experiment 5). Perfusion of the liver for five hours with the least amount of α-estradiol used (208 R.U.) led to an estrogen content in the perfusate of 15 R.U. (experiment 7) which is about the level found after perfusing the liver for three hours with no added estrogen (experiment 8). The estrogenic activity of the liver perfusate is not confined to the "estradiol" fraction but appears in both the "estrone" and "estriol" fractions (experiments 2 and 3). The estrogen of these liver perfusates appears to be in the "free" form since acid hydrolysis fails to increase the amount of activity (experiments 4 and 5). In one experiment (#4) no evidence was obtained for the potentiation of α-estradiol by the extracted perfusate.

TABLE 2. THE ESTROGEN CONTENT OF CONTROL AND EXPERIMENTAL RAT LIVERS

Experiment	Treatment	Estrogen content in R.U. per liver					Total R.U.
		Acetone extract				Liver hydrolysate	
		Total Unfractionated	Benzene fraction non-ketonic	Benzene fraction ketonic	Na_2CO_3 fraction		
—	Two fresh livers, no treatment[1]	9.9	—	—	—	18.7	28.6
8	3 hours perfusion, no estrogen[2]	[3]	—	—	—	[3]	14.1
9	3 hours perfusion, 3000 R.U. estrone	700	271	168	168	116	816

[1] Content per gram 2.68 R.U.
[2] Content per gram 2.13 R.U.
[3] The acetone and hydrolysate were combined.

The kidney perfusion apparently leads to a less rapid loss of activity, averaging 53% after three hours (experiments 13 and 14), 65% after six hours (experiments 14 and 15) and 97% after ten hours (experiment 15). The activity here appears also to distribute between the three principal fractions, but the amount appearing in the "estriol" fraction is very small, compared with the 21.6% in experiments 2 and 3.

Estrone perfusion of the liver results in a loss of activity, but at a lesser rate than when α-estradiol is used, being about 17% after three hours (experiments 9 and 10), 53% after six hours, and 78% after ten hours. Activity after estrone perfusion appears in the three principal fractions, the proportions in these fractions being roughly those found when α-estradiol is used, averaging in two sets of comparable experiments 39% "estradiol," 35% "estrone" and 21% "estriol" after estrone (experiments 9 and 10).

In a single experiment (#12) the perfusion of the liver with estriol for three hours led to a loss of 30% of the activity. It is notable that all of the activity appears in the "estriol" fraction.

In table 2 we present data on the estrogen content of certain rat livers. These data demonstrate a notable increase of estrogen in the liver perfused with estrone for three hours whereas perfusion alone causes, if anything, a decrease. Both the "free" (acetone extract) and "bound" (hydrolysate) estrogen increase, though the former is obtained in largest amount. Furthermore the "free" estrogen fractionates as "estradiol," "estrone," and "estriol."

DISCUSSION

These data indicate that in the rat liver α-estradiol is converted to a ketonic estrogen, and to a more strongly acidic phenol ("estriol").

TABLE 3. THE ESTROGEN CONTENT OF THE THREE PRINCIPAL PERFUSATE PHENOLIC FRACTIONS EXPRESSED AS MICROGRAMS EQUIVALENT OF α-ESTRADIOL, ESTRONE, AND ESTRIOL

Experiment No.	Perfusion hrs.	Estrogen added, in micrograms	Estrogen recovered			Total in perfusate	Total as % of estrogen added
			Estradiol, micrograms	Estrone, micrograms	Estriol, micrograms		
1	3	α-estradiol, 400	50	400	200	650	162.5
2	6	α-estradiol, 400	28	220	132	380	95.0
9	3	estrone, 3000	117	798	663	1578	52.6
10	6	estrone, 3000	83	400	200	683	22.7
11	10	estrone, 3000	41	252	76	369	12.3
13	3	α-estradiol, 520	110	711	41	852	164.0
14	6	α-estradiol, 520	77	500	70	647	124.4
15	10	α-estradiol, 520	6	28	12	46	8.8

In the kidney there is marked conversion to the ketonic estrogen but slight (perhaps no significant) production of "estriol." Estrone perfused through the liver appears to be converted to non-ketonic estrogen ("estradiol") and to "estriol." We have been careful to employ the micro-methods for estrogen fractionation where the quantities of active material involved seemed adequate for reliable separation. Their adequacy for urinary estrogen separation has been demonstrated (Pincus and Pearlman 1942). While there is lacking a complete demonstration of the applicability of these methods to the perfusates employed in these experiments the clear segregation of activity from liver and kidney perfusates and the lack of such segregation from the heart perfusate further substantiates the qualitative separation of estrogens.

Until the active constituents of each fraction are isolated and identified chemically we cannot, of course, be assured that they represent estradiol, estrone and estriol respectively. It is possible that rat tissue acts upon estradiol to produce ketonic estrogens which are not

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all estrone. Examination of the data from the point of view of quantitative amounts of estrogen obtained (table 3) show that in some experiments the estrogen recovery is apparently higher than the amount added (experiments 1, 13, 14). But lack of knowledge of the real nature of the obtained ketones and the fact that fractionation procedure is not 100% complete for such definite estrogenic substances as estradiol, estrone and estriol do not allow definite quantitative translation from biological units into weight units but at most rough indications of the possible conversion. Thus the probability that not only estrone but other ketones more potent than estrone as well may be obtained by α-estradiol conversion is not excluded. In this respect it should be noticed that such types of conversion with increased recovery (as expressed in micrograms) occur only in the metabolism of α-estradiol to other fractions while the reverse is not true. There is also a possibility that enhancing materials such as those described in the urine (Emmens, 1939, Pincus and Pearlman, 1941) may be present. In one experiment (#4) we could not demonstrate α-estradiol enhancing material. Unfortunately we have not made similar tests with the other estrogen fractions.

The decrease in amount of estrogen after perfusion with estrone indicated in the calculations of table 3 is in marked contrast to the data for α-estradiol perfusion. Even if we take account of the estrogen obtained by extraction of the liver (experiment 9, table 2) all of the added estrone cannot be accounted for. Since the data are consonant with the following conversion scheme for the liver:

"estradiol"$\leftrightarrows$"estrone"$\rightarrow$"estriol"$\rightarrow$"X," the contrast may be explicable on the assumption that the reaction tends to move to the right. Consequently estrone would be nearer to the inactive product ("X") of "estriol" destruction, whereas estradiol must first go through "estrone." This possibility is substantiated by (a) the large proportion of "estrone" in the α-estradiol perfusion and (b) the initial (3 hour) high "estriol" proportion in the estrone perfusion. The differences in amount of estrogen employed in the two sets of experiments make comparisons difficult, however, and only by careful quantitative comparisons and more accurate identification of the constituents of the three major fractions can a definitive analysis be made.

These data do establish that the kidney effects the conversion of α-estradiol to a ketonic estrogen. Only after ten hours however is there a marked loss of both "estradiol" and "estrone." The "estriol" of the kidney perfusate may represent either a minor conversion or segregation of small amounts of the other estrogens into the estriol fraction. This is possible since the fractionations are not 100% accurate in usual experience. Also the rate of inactivation of "estriol" does not decrease progressively as it is the case for "estradiol" and "estrone" but varies. In any event the metabolism of α-estradiol by

the kidney differs from that of the liver in the rate and sense of conversion as well as in the pace of inactivation.

Heller (1940) found that incubation of small amounts of α-estradiol (0.3) with rat liver slices led to complete loss of activity in one hour at 39°C., whereas kidney tissue reduced the activity by half. Referring to the liver he states that "the probability of a conversion to the two less active estrogens, estrone and estriol, is ruled out by the completeness of the inactivation." Our data indicate the probability of such conversion so that subsequently he (1944) attributes this to an "overflow" mechanism whereas the very small amounts he used are, he alleges, destroyed by the normal estrogen destructive process. Certainly with smaller amounts of added estrogen the loss of activity is rather rapid (experiments 6 and 7). Israel *et al.* (1937) were unable after a few minutes to recover active estrogen from dog's liver perfused with small amounts of estrone. But if we consider the rates of conversion in the scheme presented above there is no need to invoke two mechanisms; one will suffice. We cannot be certain, of course, that incubation with tissue slices and perfusion necessarily involve the same enzymatic mechanisms that presumably handle the estrogens.

Heller (1940) found conversion of estrone to other estrogens but his findings are acceptable only inasmuch as the rat uterus assay method is accurate for qualitative and quantitative determination of unknown estrogen.

It is notable that these data support our (1944) finding that the rate of conversion of estrone to "estriol" by rats in vivo is much reduced by partial hepatectomy. Removal of seven-eighths of the liver tissue resulted in an increased excretion of "estradiol," "estrone" and "estriol" after estrone injection into rats, but the amounts of "estrone" and "estradiol" increased much more than the "estriol." We concluded that tissues other than the liver were active in the estrone-estradiol conversion, and the data of this paper indicate definite kidney activity. The quantitative data in these two types of experiments differ necessarily but the qualitative data concur in showing the fundamental fact of conversion preceding inactivation. The increased estriol excretion observed after hepatectomy may be attributed to a differential reduction in the process estriol→"X" that is indicated by these perfusion data; otherwise it is possible that in addition to the conversion scheme indicated, each estrogen is subject to a secondary destructive process.

$$\begin{array}{ccccc} & & Z & & Y \\ & & \uparrow & & \uparrow \\ (2) & \text{estradiol} & \rightleftharpoons & \text{estrone} & \rightarrow \text{estriol} \rightarrow X \end{array}$$

Identification of the presumable inactive products should resolve these alternatives. Our data (Schiller and Pincus 1943a) indicating a

large excretion of estriol into human urine after its injection with contrasting smaller recoveries after α-estradiol injection might be construed to favor (2), but in human subjects we have the problem of esterification and rate of ester excretion. With rats we have found no evidence for such esterification with either *in vivo* or these *in vitro* experiments. The fact that the rat seems to metabolize exogenous estrogens in the same way as humans may explain the suitability of this animal for bio-assay.

Another interesting point is the differential behavior of the liver in respect to endogenous as compared to exogenous estrogens. The data in table 2 indicate that the liver of the ♀ rat in proestrus contains an average of about 2.5 R.U. (as estrone) per gm. of tissue. A three hour perfusion without added estrogen will not inactivate the endogenous estrogen while hundreds of R.U. of exogenous estrogen perfused during the same space of time will be inactivated. Also a five hour perfusion with 208 R.U. of α-estradiol leaves at the end of the experiment approximately the same 2.5 R.U. per gm. of tissue, and this is probably more than a coincidence. Non-inactivation of endogenous steroid hormones by the liver was shown for adrenal androgen (Burrill & Greene, 1941) adrenal estrogen (Schiller, 1944), and adrenal hormones concerned with life maintenance and water intoxication (Eversole *et al.*, 1940). Golden & Severinghaus (1938) showed that the grafting of ovaries to the mesenteries impeded the variations of the estrous cycle. This may imply that the endogenous estrogens are stored in the liver without being released in the systemic circulation perhaps not above a given threshold. Markee (1940) found a local activity of estrone pellets implanted in the anterior chamber of the monkey eye while Goodman (1934) under the same conditions found in the rat a general reaction to grafted ovaries. Their problem of differential behavior of the animal organism in respect to endogenous and exogenous steroid hormones is under investigation.

SUMMARY

Experiments are described in which rat livers were perfused with α-estradiol and estrone for varying periods of time and the estrogen content of the perfusate determined by bio-assay. There is a regular loss of estrogenic activity with increasing times of perfusion. Considerable portions of the activity are found in "estriol," "estrone" and "estradiol" fractions. After perfusion of a liver with estriol, activity appeared only in the "estriol" fraction. Perfusion of a rat heart with α-estradiol led to recovery of the activity practically exclusively in the "estradiol" fraction. With small amounts of α-estradiol, perfusion of the liver for a sufficiently long period of time leads to reduction of perfusate activity to the level of a liver perfused with no added estrogen. It is concluded that these data are consistent with a series of conversions in which: estradiol⇆estrone→estriol→X.

The perfusate of rat kidney perfused with α-estradiol also shows declining activity with time. The proportion of "estriol" in such perfusates is very low indicating a conversion to "estrone" as the principal (probably only) kidney process.

These data taken in conjunction with data in the literature indicate that the estrogens *in vivo* may be metabolized according to the scheme: estradiol⇆estrone→estriol→X.

$$\begin{array}{ccc} \downarrow & & \downarrow \\ Z & & Y \end{array}$$

The rate of estrogen turnover by the rat liver is more rapid than that of the kidneys.

The perfused rat liver retains a certain proportion of perfusing estrone, of which a small percentage is "bound." Practically all of the estrogen of the perfusate appears to be "free" i.e. unesterified.

The animal organism acts differentially upon exogenous estrogens, which are rapidly inactivated, as contrasted to endogenous estrogens which are more resistant.

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EFFECT OF PROLONGED STIMULATION OF THE ADRENAL CORTEX AND OF ADRENALECTOMY ON THE NUMBERS OF CIRCULATING ERYTHROCYTES AND LYMPHYOCYTES[1]

ABRAHAM WHITE AND THOMAS F. DOUGHERTY
From the Departments of Physiological Chemistry and Anatomy, Yale University
NEW HAVEN, CONNECTICUT

THE ROLE of the pituitary adrenotrophic hormone in the regulation of the lymphocytes and other cellular elements of the blood has been elucidated by examination of the blood picture of animals following single injections of the hormone (Dougherty and White, 1944). The action of the pituitary hormone is due to the trophic influence it exerts on the adrenal cortex. The striking alterations in blood picture seen following single injections of hormone led to examination of the effects of continued treatment with pituitary adrenotrophic factor. These results have been compared with blood changes in adrenalectomized animals.

MATERIALS AND METHODS

Mice of both sexes 60 to 80 days of age and of the CBA strain (Strong) were used. The data were similar in both sexes. Male rats (Sprague-Dawley strain) weighing approximately 200 gms at the time of adrenalectomy were used. All animals were kept at a constant environmental temperature. Purina Fox Chow and water were available at all times. The adrenalectomized rats received a solution of 0.7% NaCl and 0.3% $NaHCO_3$ as drinking fluid. In the series of adrenalectomized mice maintained on desoxycorticosterone acetate, each animal received daily subcutaneous injections of 0.025 mg. (in sesame oil, Schering) (Pfeiffer and Hooker, 1940). Purified adrenotrophic hormone (Sayers, White and Long, 1943) was injected subcutaneously in aqueous solution (1.0 mg. hormone per 0.5 ml. solution).

Total red cell and white cell determinations were made in the usual manner. Differential counts were obtained by counting at least 200 cells in air-dried blood films stained with Mäy-Grunwald Giemsa. Hemoglobin values were determined by the alkaline hematin method (Evelyn, 1936). Specific

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gravity of whole blood was determined by the falling drop method (Kagan, 1941). All analytical studies were made on tail vein blood.

RESULTS

Adrenotrophic hormone administration. Normal mice were injected daily with 1.0 mg. adrenotrophic hormone. Groups of 6 mice were studied at 3-day intervals. Since the blood picture at the first 3-day interval did not differ significantly from that at any subsequent time, the entire data are considered together in Table 1.

The values in Table 1 reveal the following effects of continued injections of adrenotrophic hormone in the normal mouse: a significant increase in hemoglobin and red cells, a persistent, absolute lymphopenia and an increase in polymorphonuclear leucocytes. The slight increase in whole blood specific gravity is probably due to an increase in both red cells and serum proteins (White and Dougherty, 1944). There was no alteration in the total number of leucocytes.

Effect of adrenalectomy. Adrenalectomized mice, not treated with desoxycorticosterone acetate, were studied in groups of 3 at daily intervals for a 5-day period. The data for all these animals are considered together in Table 1.

Adrenalectomy in the mouse is followed by the hemoconcentration observed in many species when therapeutic measures are not instituted following adrenal removal. Because of this hemoconcentration, some of the blood findings in this series of adrenalectomized mice are difficult to evaluate. However, it is apparent from Table 1 that the increased red cell count is clearly lower than would be commensurate with the markedly elevated hemoglobin value. Both the total leucocyte and lymphocyte counts are significantly increased. That this increase is not due entirely to hemoconcentration is evident from data presented below on adrenalectomized mice in which blood volume was maintained at an approximately normal level. Despite the marked hemoconcentration, the absolute number of blood polymorphonuclear leucocytes was actually somewhat less than that seen in the normal mice.

The daily administration of 0.025 mg. of desoxycorticosterone acetate to adrenalectomized mice prevents in some measure the blood concentration observed in untreated, adrenalectomized animals. In the series of animals receiving desoxycorticosterone acetate, the hemoglobin values are still elevated, although not strikingly so statistically. The red cell counts are not significantly different from normal and here again do not follow the hemoglobin trend. As in the other series of adrenalectomized mice, the total numbers of leucocytes and lymphocytes are significantly higher than in unoperated animals. There is no change in the number of polymorphonuclear leucocytes.

The effects of adrenalectomy on the blood picture of the rat are shown in Table 2. Hemoconcentration did not occur in these animals. Two days after removal of the adrenals the hemoglobin level is sig-

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TABLE 1. EFFECT OF CONTINUED INJECTION OF ADRENOTROPHIC HORMONE AND OF ADRENALECTOMY ON CERTAIN BLOOD CONSTITUENTS OF THE MOUSE[1]

	Control uninjected animals	Adrenotrophic hormone injected animals, 1 mg. daily, 15 days	P values[2]	Adrenalectomized animals	P values[2]	Adrenalectomized animals + desoxycorticosterone acetate	P values[2]
No. of animals	99	30		14		32	
Hemoglobin, gm. %	15.3 ±0.62[3]	16.4 ±0.25	<0.01	17.5 ±0.27	<0.01	15.9 ±0.18	0.06
R.B.C., mill./cu. mm.	9.05 ±0.50[3]	10.15 ±0.19	<0.01	9.73 ±0.47	<0.01	9.54 ±0.25	0.10
W.B.C.	12,814 ±435	11,749 ±737	0.25	17,025 ±1,682	<0.01	15,761 ±819	<0.01
Lymphocytes	9,584 ±371	6,932 ±510	<0.01	14,549 ±1,514	<0.01	11,674 ±684	<0.01
Polymorphonuclear leucocytes	3,257 ±158	4,694 ±358	<0.01	2,476 ±287	0.07	3,087 ±397	0.6
Sp. g. whole blood	1.060 ±0.0006[4]	1.063 ±0.0058[5]	0.05	1.068 ±0.0013	<0.01	1.064 ±0.0002	<0.01

[1] Values in this table are means and standard errors.
[2] P values as compared to controls.
[3] 39 animals.
[4] 19 animals.
[5] 10 animals.

TABLE 2. EFFECT OF ADRENALECTOMY ON CERTAIN BLOOD CONSTITUENTS OF THE RAT[1]

	Control uninjected animals	2 days after adrenalectomy	P values[4]	4 days after adrenalectomy	P values[4]	6 days after adrenalectomy	P values[4]
No. of animals	42	13		9		9	
Hemoglobin, gm. %	14.8 ±0.14[2]	14.0 ±0.35	0.01	13.4 ±0.27	<0.01	12.8 ±0.38	<0.01
R.B.C., mill./cu. mm.	8.88 ±0.23[2]	9.38 ±0.31	0.25	8.02 ±0.18	0.10	7.93 ±7.73	0.05
W.B.C.	17,826 ±809	17,194 ±1,748	0.70	19,011 ±2,016	0.50	26,082 ±1,825	<0.01
Lymphocytes	14,700 ±724	13,946 ±1,182	0.60	15,739 ±1,597	0.50	20,011 ±1,201	<0.01
Polymorphonuclear leucocytes	2,932 ±734	4,163 ±274	0.05	3,272 ±839	0.50	6,072 ±921	<0.01
Sp. g. whole blood	1.059 ± .00004[3]	1.058 ±0.001	0.80	1.057 ±0.0009	0.90	1.056 ±0.0002	0.90

[1] Values in this table are means and standard errors.
[2] Values for 27 animals.
[3] Values for 8 animals.
[4] P values as compared to controls.

nificantly lowered and is not accompanied by a decrease in number of red cells; this is still the case four days after adrenalectomy. On the sixth day, the red cell count is statistically significantly lower than that found in unoperated rats. Also on the sixth day after operation there is an increase in total leucocytes and an absolute lymphocytosis. This white cell blood picture resembles that of adrenalectomized mice. Unlike the adrenalectomized mice, the operated rats showed a significant increase in polymorphonuclear leucocytes.

DISCUSSION

Continued, daily injections of adrenotrophic hormone into normal mice produce an elevated blood hemoglobin and red cell concentration. It may be recalled that a single injection caused an initial rise in hemoglobin and red cell count within 3 hours, followed by a drop in these blood constituents so that at 24 hours after injection these values were lower than those observed in untreated animals (Dougherty and White, 1944). It is possible that repeated hormone injection eventually leads to a stimulated production of red cells in an effort to compensate for the diminution of erythrocyte count produced by a single dose of hormone. Histological evidence is available (Dougherty and White, unpublished) supporting the suggestion that continued hormone administration stimulates red cell production. Both in man and in experimental animals, the presence in the organism of increased amounts of either anterior pituitary secretion (Neuberger, 1927; Flaks, Himmel and Zlotnik, 1937, 1938) or of adrenal cortical hormones (Günther, 1929; Huth, 1929; Zucker, 1929; Brown, 1930; Moehlig and Bates, 1933) results in polycythemia. Polycythemia also occurs in Cushing's syndrome (Günther, 1929; Cushing, 1932). Conversely, anemia is present clinically in Simmond's disease (Silver, 1933) and is frequently observed in Addison's disease (Hartman, Greene, Bowen and Thorn, 1932). Experimentally, it is well established that hypophysectomy is followed by an anemia (Aschner, 1912; Houssay, Roger, and Orias, 1931; Meyer, Stewart, Thewlis and Rusch, 1937; Vollmer, Gordon, Levenstein and Charipper, 1939; Crafts, 1941).

Since pituitary adrenotrophic hormone administration produced an increased red cell and hemoglobin concentration, a variety of stimuli which are known to increase pituitary-adrenal cortex secretory activity might cause a polycythemia. This suggestion finds considerable support in the literature. The polycythemia of anoxia does not occur in hypophysectomized rats 10 days after operation (Meyer, Stewart, Thewlis and Rusch, 1937). Anoxia is a stimulator of the pituitary-adrenal cortex mechanism (Evans, 1934, 1936; Darrow and Sarason, 1944). Further, several of the stimuli which augment red cell and hemoglobin concentration also cause increased adrenal cortical secretion. For example, hemorrhage, arsenic (Wintrobe, 1942),

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epinephrine (Davis, 1942), posterior pituitary extract (Davis, 1942), and insulin (Drabkin and Edwards, 1924; Levine and Kolars, 1925; Torok, 1925), stimuli which cause increased red cell production, also bring about increased adrenal cortical secretion as measured by adrenal cholesterol concentrations or by depletion of adrenal cortical sudanophilic material (Sayers, Sayers, Fry, White and Long, 1944; Dougherty and White, unpublished).

The erythrocytosis in the adrenotrophic hormone injected animals and their increased serum protein levels (White and Dougherty, 1944) are reflected in the increased whole blood specific gravity.

Adrenalectomy in the mouse, unattended by therapy to prevent hemoconcentration, produced a marked increase in hemoglobin and red cells due to hemoconcentration. When the marked degree of hemoconcentration is partially prevented in adrenalectomized mice by daily injections of desoxycorticosterone acetate, there is no significant rise in either hemoglobin concentration or red cell counts. However, despite the fact that the whole blood specific gravity in adrenalectomized mice receiving desoxycorticosterone acetate was identical with that seen in normal mice given daily injections of adrenotrophic hormone, the adrenalectomized animals had a normal red cell and hemoglobin picture while the injected group showed an erythrocytosis. Therefore, the adrenalectomized mice actually had a decreased red cell production. This is substantiated in the data for adrenalectomized rats 6 days following operation.

To recapitulate, a pituitary-adrenal cortical role in either the production or delivery of red cells, or perhaps both, is indicated by the following observations: 1) the increase in red cells and hemoglobin in normal mice treated with adrenotrophic hormone, 2) the significant decrease in hemoglobin and red cell count in adrenalectomized rats having a normal whole blood specific gravity, and 3) the failure of adrenalectomized mice to show elevated red cell counts despite a reduced plasma volume.

The hemoconcentration occurring in adrenalectomized mice, despite desoxycorticosterone acetate injection, could well be due to the failure of this steroid to affect lymphoid tissue (White and Dougherty, 1944) and thereby prevent the fall in total serum proteins seen in adrenalectomized mice.

A single injection of adrenotrophic hormone in normal mice produces a lymphopenia which is most profound at 9 hours following injection. Shortly thereafter the blood picture begins to return to normal and the level of blood lymphocytes is almost normal 24 hours after injection of hormone (Dougherty and White, 1944). In the present study, repeated hormone injection at 24 hour intervals kept the blood lymphocytes consistently at a level lower than normal. This level is slightly lower than that seen in animals 24 hours after one injection of hormone. Despite the lower lymphocyte count with con-

tinued hormone injection, the total white counts remain approximately normal because of the concomitant increase in polymorphonuclear leucocytes. The increase in polymorphonuclears is not a specific hormone effect because it occurs following the injection of adrenotrophic hormone in adrenalectomized animals (Dougherty and White, 1944).

Adrenalectomy removes the humoral agents which normally regulate the dissolution of lymphocytes (White and Dougherty, 1944). A lack of this normal control results in the increased total lymphocyte count (Dougherty and White, 1944) and a hyperplasia of lymphoid tissue (Reinhardt and Holmes, 1940; Dougherty and White, unpublished). Some maturation and disposal of lymphocytes must continue in the absence of the adrenal cortex, but at a greatly diminished rate Dougherty and White, unpublished). An increase in blood lymphocytes following adrenalectomy occurs in other species (Zwemer and Lyons, 1928; Corey and Britton, 1932). A lymphocytosis is frequently observed in Addison's disease (Grollman, 1936).

Following adrenalectomy, no increase in polymorphonuclear cells was noted in untreated animals or in mice given desoxycorticosterone. This finding is interesting in view of the fact that although there is a decreased blood volume, there is no relative rise in these cells. Therefore, it would seem that there is an actual suppression in the production or in the delivery of polymorphonuclear cells following adrenalectomy. Similar data have been reported by Corey and Britton (1932) in adrenalectomized cats.

Although in these studies continued hormone injection produced a persistent lymphopenia, chronic adrenal cortical stimulation need not necessarily give a lymphopenic picture. It is not unlikely that continued accelerated lymphocyte dissolution is an incitant of lymphocyte production. This production may proceed at a rate greater than that of lymphocyte dissolution even though the latter process may be continuing more rapidly than normal. Thus the stimulus initially responsible for a lymphopenia may, if continued, cause a lymphocytosis. The production of a lymphocytosis could result from the gradual exhaustion of the adrenal cortex due to its prolonged stimulation. In this event, there would be a diminishing supply of humoral agents concerned with lymphocyte disposition, and the blood lymphocyte picture would alter in the direction of resembling that seen in an adrenalectomized animal.[2]

It is to be emphasized that the relationship obtaining at any time between two factors, namely, lymphoid tissue proliferative capacity, and supply of adrenal cortical hormones, will determine the nature of

[2] This is a theoretical possibility. However, unpublished studies indicate that in mice injected with adenotrophic hormone daily for a period as long as one year, a persistent, absolute lymphopenia is present. However, toxic adrenal cortical stimulants might be expected more readily to cause exhaustion of the adrenal cortex due to toxic injury of this tissue.

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the blood lymphocyte picture. Lymphoid tissue proliferation may be influenced by the supply of tissue components in the diet, by metabolic activity and perhaps by unknown hormonal factors. Supply of adrenal cortical hormones is dependent on pituitary influence and on available hormone in the adrenal cortex. These, in turn, are affected by numerous circumstances (Sayers, Sayers, Fry, White and Long, 1944).

Several examples may be cited as illustration of the above explanation of blood lymphocyte levels. Acute infectious processes, inanition (Emmel and Streicher, 1929; Shukers and Day, 1943), insulin (Latta and Henderson, 1937), anoxia (Meyer, Seevers and Beatty, 1935; Isihara, 1938), X-ray (Taylor, Witherbee and Murphy, 1919; Mottram and Russ, 1921; Mottram, 1931; Latta and Ehlers, 1931; Henshaw, 1944), bacterial toxins (Conway, 1938), arsenic (Halter, 1936; Beitschinger, 1937), cold (Dougherty and White, unpublished), and a variety of other circumstances induce a lymphopenia. These same stimuli also cause augmented adrenal cortical secretion (Sayers, Sayers, White, Fry and Long, 1944; Dougherty and White, unpublished). However, continued exposure to many of these same stimuli alters the lymphopenic picture to one of lymphocytosis.

SUMMARY

Continued daily injection of adrenotrophic hormone into mice resulted in an increase in hemoglobin and in red cells, an absolute lymphopenia and an increase in polymorphonuclear leucocytes. There is a trend toward an anemia in adrenalectomized rats and mice. The operated animals also show an absolute lymphocytosis and a depressed polymorphonuclear production. By means of its trophic influence on the adrenal cortex, pituitary adrenotrophic hormone maintains a physiological control over the numbers of circulating erythrocytes and lymphocytes. Alterations in the numbers of these cells seen following a wide variety of unrelated stimuli may be explained in part by the fact that pituitary adrenal cortical secretion is also responsive to these same stimuli.

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THE MAINTENANCE OF MUSCLE GLYCOGEN IN FASTED HYPOPHYSECTOMIZED-ADRENALECTOMIZED RATS[1]

LESLIE L. BENNETT AND ROBERT Z. PERKINS

From the Divisions of Physiology (Berkeley) and Medicine (San Francisco), Medical School, and the Institute of Experimental Biology (Berkeley)

UNIVERSITY OF CALIFORNIA

SINCE THE ORIGINAL REPORT (Russell and Bennett, 1936) that crude anterior pituitary extracts maintained the muscle glycogen levels of fasted hypophysectomized rats even in the absence of the adrenal cortex, evidence has been presented that adequate doses of adrenal cortical steroids had a comparable effect and also elevated blood glucose and liver glycogen levels (Russell and Craig, 1938; Long and Katzin, 1938). In addition Russell (1940) has reported that in adrenalectomized rats the maximal suppression of the oxidation of fed glucose could be obtained with anterior pituitary extracts only when cortical steroids were also present. Since in the original observations on hypophysectomized-adrenalectomized rats the sequence of operations was such that the animals were sacrificed only 24–28 hours after completion of their bilateral adrenalectomy, they never developed adrenal cortical insufficiency and presumptively cortical steroids may still have been present in their body fluids. It therefore seemed to be important to alter the experimental conditions so that the question could be answered as to whether the presence of traces of adrenal cortical hormone was essential for the "myoglycostatic" effect of crude anterior pituitary extract.

METHODS

In the present series of experiments two types of anterior hypophyseal extract were used. The extract designated SAE was a crude alkaline extract prepared according to the method of Evans and co-workers (1933) and was rich in growth-promoting and myoglycostatic effects. The extract designated ACTH[2] was a highly potent and biologically pure adrenocorticotropic hormone. In every case the extracts were administered intraperitoneally, and were given only during the fasting period as indicated. Hypophysectomies were done by the parapharyngeal approach and the completeness of the

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operation was checked at autopsy by inspection of the sella through a binocular dissecting microscope. Adrenalectomies were done through a bilateral lumbar approach and the region of the adrenal was inspected for accessory cortical tissue or cortical remnants at autopsy.

In the doubly operated animals the sequence of operations was carried out in two different ways. In those designated H-A-3, a right adrenalectomy and a hypophysectomy were done on the same or on subsequent days and then 13–14 days later the left adrenal was removed and fasting was instituted immediately. In those designated as H-A-2 a bilateral adrenalectomy was done and then the animals were maintained on the stock diet until they developed signs of adrenal cortical insufficiency as judged by a significant lowering of body temperature plus a failure to gain weight normally. They were then placed on a substitution therapy consisting of 1 per cent sodium chloride solution ad libitum as drinking water. When they had shown a satisfactory weight gain (20–80 gm. over 6–24 days) they were hypophysectomized and fasting was immediately instituted. This procedure insured the use of rats in good nutritional state and took advantage of the fact that as soon as 24 hours after hypophysectomy the typical inability of a hypophysectomized rat to maintain its fasting glycogen stores is present (Russell and Bennett, 1937).

Analytical procedures were carried out as reported previously (Russell and Bennett, 1937).

RESULTS AND DISCUSSION

The significant data are presented in the accompanying table. Inspection of the data shows that we have again confirmed the fact that

TABLE 1. CARBOHYDRATE LEVELS OF RATS FASTED FOR 24 HOURS

	Blood glucose mg. per cent		Liver glycogen mg. per cent		Muscle glycogen mg. per cent	
	Mean	Range	Mean	Range	Mean	Range
Normal Controls (13)[1]	77	65–91	20	9–46	465	413–509
Hypophysectomized Controls (10)	63	55–72	14	11–22	298	196–360
Hypophysectomized plus SAE[2] (6)	50	43–60	13	11–16	470	398–526
Hypophysectomized plus ACTH[3] (5)	63	58–69	10	7–15	320	255–387
H-A-3[4] plus SAE[2] (6)	50	26–70	16	12–19	483	426–513
Adrenalectomized Controls (6)	59	44–68	16	8–35	475	454–505
H-A-2[4] Controls (6)	55	46–66	14	8–29	352	273–397
H-A-2[4] plus SAE[2] (6)	58	27–68	14	12–16	443	390–507

[1] Number of animals in the group.
[2] Standard alkaline extract. A total dose of 9 mg. given intraperitoneally in divided doses at 24, 19–20, and 4 hours prior to sacrifice.
[3] Adrenocorticotropic hormone. A total dose of 2.4 mg. given in divided doses each 2 hours during fasting.
[4] Hypophysectomized-adrenalectomized rats. See text for sequence of operations.

the muscle glycogen levels of fasted hypophysectomized rats are significantly below the normal levels. The hypophysectomized rats had an average muscle glycogen value of 298 mg. per cent as compared with 465 mg. per cent for the controls. There was no overlapping of

the individual figures in the two groups. Use of a crude alkaline extract (SAE) resulted in complete maintenance of muscle glycogen at normal levels without comparable effects on blood glucose or liver glycogen. Likewise, complete maintenance of muscle glycogen was obtained when the remaining adrenal was removed at the beginning of the fasting period (H-A-3). However, as pointed out previously the sequence of operations was such that cortical steroids were still present at the time of the first injection of SAE and may have been present until the time of sacrifice. In contrast with these findings the ACTH was without significant effect on the muscle glycogen levels under the experimental conditions here employed. Similar observations have previously been reported (Herring and Evans, 1943).

The data further show that adrenalectomized rats proven to have had adrenal cortical insufficiency and then maintained on sodium chloride substitution therapy had completely normal muscle glycogen stores. The mean was 470 mg. per cent as compared with the normal control value of 465 mg. per cent. This fact in itself speaks against the necessity for the presence of adrenal cortical hormone. When hypophysectomy was added to adrenalectomy the typical inability of the hypophysectomized rat to maintain normal muscle glycogen stores during fasting appeared (H-A-2). Again this defect could be repaired by the use of SAE, and in these experimental conditions there could be no possible participation of adrenal cortical hormones.

SUMMARY

A crude alkaline extract of the anterior pituitary when given only during the fasting period maintained normal muscle glycogen levels of 24-hour fasted, hypophysectomized, and hypophysectomized-adrenalectomized rats. Pure ACTH was without effect. The presence of adrenal cortical hormones was not necessary for the "myoglycostatic" effect, as complete maintenance of muscle glycogen stores could be obtained in animals proven to have adrenal cortical insufficiency and maintained for long periods of time on salt prior to hypophysectomy.

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STUDIES ON THE EFFECTIVENESS OF DESOXYCORTICOSTERONE ACETATE IN ADRENALECTOMIZED RATS GIVEN A LOW SODIUM CHLORIDE DIET

WILBURN J. EVERSOLE
From the Department of Biology, The Rice Institute
HOUSTON, TEXAS

IT IS WELL KNOWN that a high sodium chloride intake can substitute in part for the hormones of the adrenal cortex. Of the crystalline adrenal steroids desoxycorticosterone, particularly, is concerned with electrolyte metabolism in the body (references cited by Swingle and Remington, 1944). A somewhat different aspect of this action of desoxycorticosterone was suggested by the report of Grollman (1941a) that this steroid was relatively incapable of exerting its characteristic life-maintaining effect in adrenalectomized rats when these animals were fed a low salt diet.

This report concerns a further study of the action of desoxycorticosterone acetate in adrenalectomized rats on a diet almost completely free of sodium chloride.

METHODS

Three different diets, besides our stock diet, were used for testing the effectiveness of desoxycorticosterone on growth and survival in young adrenalectomized rats. Diet 1 was the same as that recommended by Grollman (1941b) for securing uniform results in a method for the biological assay of adrenal cortical steroids. The salt content of this diet probably is not markedly reduced from that of most stock mixtures. Diets 2 and 3 were modifications of a purified diet, virtually free of sodium chloride, suggested by R. J. Harrison of the University of Texas (Personal communication). The composition of diet 3 per kilogram was as follows:

	gm.
Dextrose	743
Vitamin-test casein	180
Salts	
Magnesium sulphate	5.5
Potassium phosphate	9.5
Calcium phosphate	5.7
Iron citrate	1.2
Calcium lactate	13.0
Potassium iodide	.7

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Manganous sulphate	4.5
Zinc sulphate	.22
Copper sulphate	.28
Oils	
Cod liver oil	9.4
Linseed oil	5.0
Wesson oil	15.0
Wheat germ oil	3.0
Cystine	3.0
Crystalline vitamins	mg.
Thiamin hydrochloride	20
Riboflavin	10
Pyridoxine hydrochloride	10
Calcium pantothenate	30
Nicotinic acid	110
Choline chloride	300
Ascorbic acid	120
Inositol	200
Para-aminobenzoic acid	200

Diet 2 consisted of all the ingredients of diet 3 with the exception that a rice bran concentrate, supplemented with 5 mg. of riboflavin, was substituted for the crystalline vitamins. The stock diet was prepared by mixing the following:

	lbs.
Whole wheat meal	67
Alfalfa leaf meal	10
Fish meal	10
Casein	5
Sodium chloride	1
Calcium salts	1
Sardine oil	5

The animals were prepared in a manner similar to those used in previous studies on desoxycorticosterone administration (Eversole and Gaunt, 1943). Briefly, young male rats were segregated soon after weaning into groups of four or less and allowed to reach a weight of approximately 55 gm. They were then adrenalectomized, given an appropriate diet, and injected subcutaneously with 0.2 mg. of desoxycorticosterone in oil daily for 10 or 20 days.

In the majority of the experiments the rats were fed a stock diet until adrenalectomy and then changed to a salt deficient diet for a 10 day experimental period, and in most cases until death. Two sets of experiments were devised so that treated rats were subjected to a salt deficient diet for at least 20 days. In one of these experiments the rats were primed with diet 3 for ten days before adrenalectomy, and in the other, they were adrenalectomized, fed diet 3, and treated with desoxycorticosterone for 20 days, or until death.

RESULTS

Diet 1.

Adrenalectomized rats injected with desoxycorticosterone and fed

diet 1 did not respond quite as well as did similar rats on our stock diet. The reason for this difference is not known.

Diets 2 and 3.

In order to eliminate as nearly as possible sodium chloride from the diet it was necessary to prepare a purified diet consisting primarily

TABLE 1. AVERAGE FIGURES SHOWING RESPONSE OF INTACT AND ADRENALECTOMIZED RATS ON THE STOCK DIET AND DIET 1 TO 0.2 MG. DESOXYCORTICOSTERONE ACETATE GIVEN SUBCUTANEOUSLY DAILY FOR 10 DAYS.

Treatment	Diet	Number animals	Weight change during 10 day period gm./day	Survival days	Number animals died during 10 day period	Drinking fluid
Intact control	Stock	10	+5.2	Indefinite	none	Tap water
Adrex.	Stock	13	+0.7	8.1	10	Tap water
Adrex., DCA	Stock	9	+4.2	24.0	1	Tap water
Adrex.	No. 1	5	−1.8	6.2	5	Tap water
Adrex., DCA	No. 1	6	+3.8	16.5	none	Tap water

TABLE 2. AVERAGE FIGURES SHOWING RESPONSE OF INTACT AND ADRENALECTOMIZED RATS ON DIETS 2 AND 3 TO 0.2 MG. DESOXYCORTICOSTERONE ACETATE GIVEN SUBCUTANEOUSLY DAILY FOR 10 DAYS.

Treatment	Diet no.	Number animals	Weight change during 10 day period gm./day	Survival days	Number animals died during 10 day period	Drinking fluid
Intact control	2	8	+2.9	Indefinite	none	Tap water
Adrex., DCA	2	15	+1.1	12.5	1	Tap water
Intact control	3	8	+2.0	Indefinite	none	Dist. water
Adrex., DCA	3	12	+2.4	14.7	1	Dist. water
Adrex., DCA*	3	11	+1.5	13.4	2	Dist. water
Adrex., DCA*	3	11	+1.8	13.0	1	1% NaCl

* Animals fed diet 3 from shortly after weaning until end of treatment.

of synthetic ingredients. Diets 2 and 3 were of this type. All control and treated animals on these diets showed significant weight gains and the great majority of the operated animals survived the experimental periods. Treated, adrenalectomized rats on diet 2 showed, however, an average weight gain less than one-half that of intact rats on the same diet. This rather marked difference in response may have been due to a vitamin as well as a sodium chloride deficiency, since both groups of rats showed loss of hair by the end of the 10 day experimental period. The adrenalectomized animals were seemingly more sensitive to the vitamin deficiency, as indicated by extensive loss of hair and decreased appetite, resulting in a decreased gain in weight. Nevertheless, all but one of the 15 adrenalectomized animals survived the 10 day treatment, and all gained an appreciable amount of weight, thus indicating that desoxycorticosterone was effective under a combination of vitamin and salt deficiency.

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Intact rats on diet 3 showed no signs of vitamin deficiency, but growth was inferior to that on a stock diet. Adrenalectomized, treated rats fed diet 3 gained weight as well as intact control rats on the same regime. The effectiveness of desoxycorticosterone here was definitely not impaired in the almost total absence of sodium chloride intake.

That the animals on diet 3 contained sufficient stored sodium chloride in their tissues to tide them over a 10 day experimental period seemed quite possible. Two groups of rats were, therefore, given the diet for 20 days. The first group was given diet 3 for 10 days previous to adrenalectomy and for 10 days during the period of treatment. The second group (Table 3) was adrenalectomized and fed the diet for a 20 day period of treatment. The average gain in weight of the

TABLE 3. AVERAGE FIGURES SHOWING RESPONSE OF RATS TO DIET 3 AND 0.2 MG. DESOXYCORTICOSTERONE GIVEN SUBCUTANEOUSLY DAILY FOR 20 DAYS.

Treatment	Animals	Weight change during 20 day period gm./day	Survival days	Number Animals died during 20 day period	Drinking fluid
Intact controls	8	+2.1	Indefinite	none	Dist. water
Adrex., DCA	7	+1.7	24.4 (18–34)	5	Dist. water

two groups was about the same (approximately 1.5 gm./day). Nine of 11 animals survived the experimental period in the first group and all 7 animals in the second group lived at least 18 days with an average survival of 24.4 days. These results indicated that desoxycorticosterone was effective for approximately 20 days, but that the full effectiveness could not be continued on this diet indefinitely. Desoxycorticosterone was only slightly, if any, more effective when adrenalectomized rats were given 1% sodium chloride (instead of distilled water) and diet 3. The reason for the latter result is not clear and is being investigated further, but the diet was apparently lacking in something other than sodium chloride needed particularly by adrenalectomized animals.

DISCUSSION

It is clear that desoxycorticosterone acetate is effective for relatively long periods of time in the adrenalectomized rat fed a diet essentially free of sodium chloride. The growth rate was not as good as that seen when desoxycorticosterone was given with a balanced stock diet, but the salt-deficient diets also produced deficient growth in intact controls, and the results suggest that dietary deficiencies other than that of sodium chloride may be of primary importance in determining the effectiveness of desoxycorticosterone. In fact the addition of sodium chloride was not helpful in one series. The possibility that the adrenalectomized rats were receiving insufficient quantities of

vitamins is suggestive since Sandberg and Perla (1936) have presented evidence for the need of extra vitamins in adrenalectomized animals. Whether a similar interpretation can be given the work of Grollman (1941a) in which he concluded that desoxycorticosterone was relatively ineffective on a low sodium chloride intake is not known, because the constituents of his low salt diet were not stated. While there is no question that the effectiveness of cortical hormones is enhanced by concomitant salt therapy, there is little in our results to indicate that the essential actions of desoxycorticosterone in small doses cannot be demonstrated on a low salt diet.

SUMMARY

Small daily doses of desoxycorticosterone acetate maintained life and growth in adrenalectomized rats fed purified diets virtually free of sodium chloride. The effectiveness of the steroid in maintaining such animals in a healthy condition was somewhat reduced by 20 days, but it is probable that deficiencies other than those of sodium chloride were also involved.

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THE OXYGEN CONSUMPTION AND HISTOLOGY OF THE THYROID GLAND *IN VITRO*

R. S. TURNER AND M. L. TURNER[1]
From the Department of Anatomy and Department of Physiology, School of Medicine
STANFORD UNIVERSITY, CALIFORNIA

THE HISTOLOGY of the thyroid gland in normal, pathological and experimental states has been widely studied by a large number of investigators. Observations are also available on the oxygen consumption of the gland *in vitro*. Cytological study of thyroid tissues from Warburg respirometers, however, has been almost wholly neglected. In order to validate microscopical observations made on such material it is necessary to demonstrate that the Warburg technique introduces no significant distortion of intracellular architecture or that any distortion which does occur is constant under given conditions. It is believed that the experiments to be reported provide such a demonstration. If such a cytological base may be regarded as established, the technique presents unique advantages for the correlation of controlled functional states with thyroid histology. It is possible to control the environmental temperature of the tissue, to inhibit specific enzyme systems, to alter the ionic balance, composition and concentration of the substrate, or to initiate other procedures and thus to correlate the functional activity with the histological picture. Control portions may be selected from the same lobe that is used in the respirometer and compared histologically with portions of the gland used in the oxygen consumption determinations.

QO_2 values for the thyroid heretofore reported in the literature have been variable and unreliable since they were necessarily calculated on a weight basis and the proportion of this weight contributed by colloid was not accurately estimated. Proper application of photographic and planimetric techniques to histological preparations can in great part overcome this difficulty.

The position and appearance of the Golgi apparatus were selected for study in the present investigation because this structure has already received considerable attention in the literature on the thyroid gland, and because alterations in its integrity are usually taken as an index of cell trauma.

In this series of experiments an attempt has been made to relate

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oxygen consumption to certain aspects of the histology of the thyroid gland.

MATERIALS AND METHODS

Mature male guinea pigs weighing approximately 350 grams were used in the experiments. After the animals were killed by decapitation, the thyroid glands were quickly removed to a cold box saturated with water vapor (Fuhrman and Field, 1944). Very thin slices of gland were then made with a pair of sharp iridectomy scissors. Control slices from both lobes of the gland were immediately placed in appropriate fixatives. The remaining slices were then placed in the Warbug vessels in Dickens-Greville Ringer solution buffered to a pH of 7.2. One half of one lobe of the gland was usually run at 5°C. and the remaining half of the same lobe at 40°C. The entire opposite lobe of the gland from the same animal was usually run at 38°C. Precautions suggested by Dixon (1943) were observed. The total time elapsing between removal of the gland from the animal and the beginning of the run was ordinarily around twenty minutes. At the end of seventy-five minutes in the respirometer the tissues were removed from the vessels and placed in fixative. From this point on, the histological treatment of the control and experimental tissues was identical. For studies of the Golgi apparatus the technique of Kolachev-Nassanov was followed, with sections cut at 4 micra. In order to calculate cell area and colloid area, photographs of entire typical sections of the glands were taken at a constant low magnification through a compound microscope. Enlargements to 280 diameters were then made on matte surface paper and total follicle-area and follicular-lumen area were measured directly with a Coradi #2210 planimeter set to read in square inches. Cell area was obtained by subtraction. The cell area for each follicle was then converted into cell volume and the mean volume calculated for the entire gland. This procedure was adopted in order to avoid the error introduced if mean areas are converted directly into mean volumes. The calculation of ratios of cellular area to follicular lumen area must also take into account consideration of individual follicles.

OBSERVATIONS

Data showing the effect of temperature on the oxygen consumption, cellular areas and cellular volumes of the thyroid gland *in vitro* are presented in Table 1. The determinations of area and the calculations of volume at 38° C. were based upon a typical sample from animal T2; those at 5° C. and 40° C. were made from the typical sections illustrated in Figures 1 and 2. In obtaining the cellular weight by subtraction of colloid weight from total weight, the colloid was assumed to have a specific gravity of one. It may be noted that, either on the basis of cc. O_2/gram wet wt. of gland/hr. or on the basis of cc. O_2/gram *cellular* wet wt./hr., there is an increase in oxygen uptake with an increase in thermostat temperature. The discrepancy between the values calculated by these two methods also increases with increase in temperature. This discrepancy may be due in part to a true change in cellular volume at the various temperature levels

The mean QO_2 values for all samples of thyroid used in these ex-

periments are to be found in Table 2. The difference between the mean QO_2 at 5° C. and 40° C. and at 5° C. and 38° C. is highly significant. The P value in these cases is less than 0.0000001—i.e. such a difference would be expected to occur by chance alone less than once in ten million times (Fisher, 1936). The P value for the difference between the means at 38° C. and 40° C. is also statistically significant, lying between 0.01 and 0.001. In Table 2 the values for mean oxygen consumption in terms of cc. O_2/gram cellular wet wt./hr. were based on the percentage cellular volumes in Table 1.

Selected fields showing the Golgi apparatus in the control piece of

TABLE 1. EFFECT OF TEMPERATURE ON QO_2, CELLULAR AREA AND CELLULAR VOLUME

	5° C.	38° C.	40° C.
Animal number	T4	T2	T4
No. follicles examined	40	40	40
Mean cell area	0.255	0.176	0.134
Mean colloid area	0.230	0.225	0.290
Mean $\frac{\text{cell}}{\text{colloid}}$ ratio[1]	1.372	1.051	0.530
S.E. of mean ratio	0.132		0.048
Total cell volume[1]	7062.52	5079.09	3854.07
Total colloid volume[1]	3598.06	4547.29	5284.11
% cellular volume	66.25	52.76	42.17
% colloid volume	33.75	47.24	57.83
QO_2 (cc/gm wet wt/hr)	0.074	0.439	0.814
QO_2 (cc/gm cell wet wt/hr)	0.104	0.831	1.929

[1] Calculated as described under Materials and Methods.

TABLE 2. EFFECT OF TEMPERATURE ON OXYGEN CONSUMPTION

	5°C.	38°C.	40°C.
No. of samples	10	18	12
Mean QO_2 (cc/gm wet wt/hr)	0.103	0.547	0.797
S.E. of mean	0.021	0.034	0.096
Mean QO_2 (cc/gm cell wet wt/hr)[1]	0.156	1.037	1.889

[1] Calculated as described in text.

thyroid tissue from animal T4 (as a typical example), as well as fields showing the apparatus in pieces of thyroid from the same animal after treatment in the respirometer at 5° C., 38° C. and 40° C. are shown in Figures 3–6. Little significant difference is noted between the control sample and those run at 38° C. insofar as total amount and position of the Golgi material are concerned. As will be seen (Figs. 3–4), the Golgi apparatus is in the position and arrangement classically described for "normal" thyroid tissue; the network caps the apical side of the nucleus with some extension along the lateral nuclear surfaces. In the 5° pieces (Fig. 5) the apparatus appears increased in size, the branches of the network appear thicker, and the apparatus occupies a more supranuclear position in the cell. More

"loose" Golgi material is present in the dome-shaped apical ends of the cells. These apparently fragmented pieces of Golgi material may in reality connect to form a network, as is the case in instances of

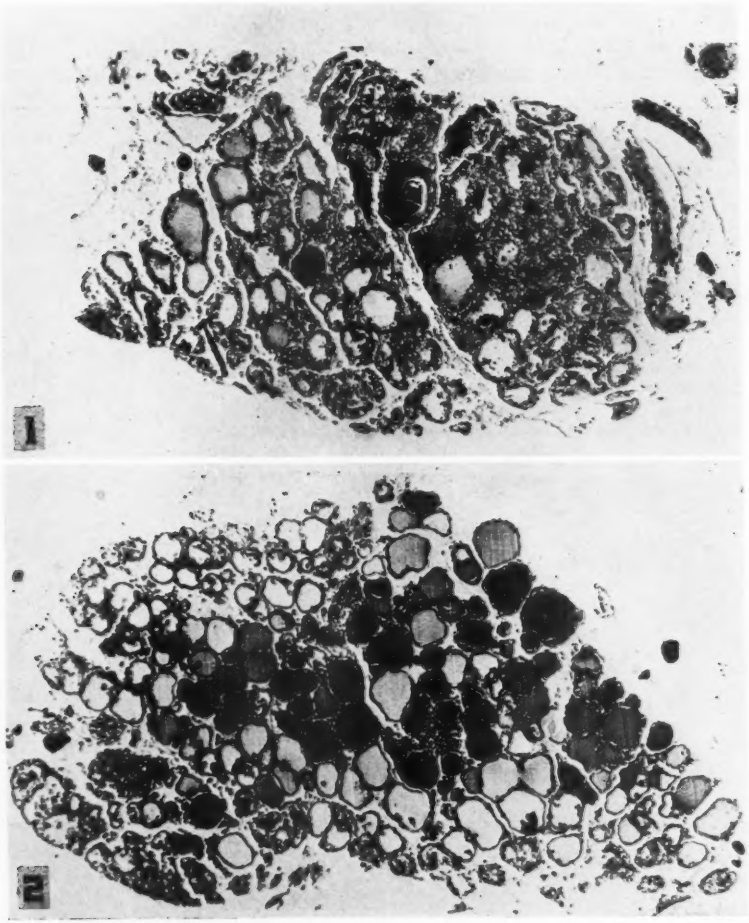

FIG. 1. Thyroid from animal T4 after Warburg run at 5° C. 80×.
FIG. 2. Thyroid from animal T4 after Warburg run at 40° C. 80×.

"fragmented" Golgi networks in spinal ganglion cells (Kirkman, 1944). In the 40° sections the Golgi apparatus appears to be reduced in total amount and tends to be located lateral to and around the nucleus, sometimes even appearing to extend for short distances beneath the basal pole (Fig. 6). No cases were observed in which the entire apparatus was located beneath the basal pole of the nucleus as

reported by Cowdry (1922), Buño and Grosso (1935) and DeRobertis (1941).

Sections of the same lobe of the same gland run in the respirometer at 5° C. and at 40° C. in most cases reveal striking differences in the height of the follicular cells, as shown for example in Figures 1 and 2. When the means of the ratios of cellular area to follicular lumen area in these two sections are compared, the difference is found to be a highly significant one. The P value in this case is less than 0.0000001.

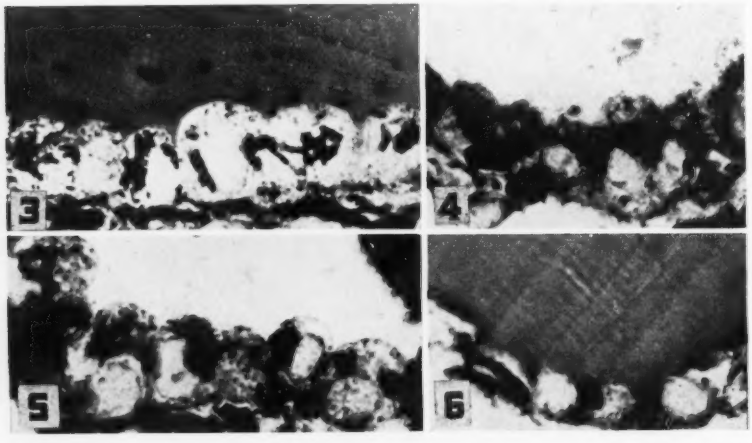

FIG. 3. Golgi apparatus in control piece of thyroid from animal T4. 1300×.
FIG. 4. Golgi apparatus in sample from animal T4 after Warburg run at 38° C. 1300×.
FIG. 5. Golgi apparatus in sample from animal T4 after Warburg run at 5° C. 1300×.
FIG. 6. Golgi apparatus in sample from animal T4 after Warburg run at 40° C. 1300×.

The figures on which these calculations are based are to be found in Table 1.

DISCUSSION

That the oxygen consumption of the thyroid gland should fall with a decrease in temperature and should rise with an increase in temperature is not surprising in view of the data on other tissues (Cf. Field, Fuhrman and Martin, 1944). It is apparent from the present data that an increase in QO_2 of the thyroid gland need not be associated with an increase in cell height (Fig. 6). Similarly, thyroid tissue which appears histologically "activated" may have a very low metabolic rate (Fig. 5). The fact that certain investigators have demonstrated an increased QO_2 *in vitro* by the addition of thyrotropic hormone to the medium (Canzanelli and Rapport, 1938; Galli-Mainini, 1941) and the fact that thyrotropic hormone administered *in vivo* is known to produce an increase in thyroid cell height has often led to

the erroneous impression that QO_2 and cell height invariably increase together.

As to the actual values for QO_2 of thyroid tissue, the figures in Table 2 are in essential agreement with those reported by other investigators, if appropriate conversion is made from dry to wet weight determinations (Canzanelli and Rapport, 1938; Stephens and Belasco, 1940; Belasco and Murlin, 1941; Vanderlaan, Vanderlaan and Logan, 1941).

The estimation of thyroid weight by subtraction of calculated colloid weight from total weight of the sample does not evaluate progressive cellular change during the Warburg run nor the proportion of the weight contributed by blood vessels and connective tissue. Since, however, equivalent samples of thyroid tissue of the same species under comparable conditions would be expected to contain equivalent amounts of this extraneous material, any error introduced into QO_2 determinations would be a relatively constant one. As most investigations of the QO_2 of the thyroid gland are done under conventionally standardized conditions, consideration of actual cellular volumes is not usually essential for reliable comparisons between data of different workers. However, in the case of glands activated by thyrotropic hormone, cold, or other procedures, the actual change in cellular to colloid ratio may be a matter of real importance in the calculation of valid oxygen consumption values.

Conclusions as to the rôle of the Golgi apparatus in the function of the thyroid cell can perhaps best be evaluated after further studies have been carried out, as, for example, with the use of enzyme inhibitors, iodine, thiouracil, or other variations in the substrate. With regard to the histological interpretation of the material reported here it might be that the changes observed in the position of the Golgi apparatus merely represent a physical adaptation to the space available in the cell, a possibility strongly discounted by Severinghaus (1933). If total volume of Golgi material be taken as an essential criterion (Bowen, 1926), reconstructions (Gillman, 1934) may be necessary to determine the volume of the apparatus within given cells. Moreover, since great variability in Golgi apparatus is usually present in sections, extreme care must be exercised in selection of fields for study. Despite a large body of literature dealing with the subject, there still seems to be no general agreement as to standards of normality of the Golgi apparatus (Bowen, 1926; Dalton, 1934; Kirkman and Severinghaus, 1938; Pollister, 1939), and quantitative errors may also be introduced during the impregnation procedures (Owens and Bensley, 1929; Gatenby, 1931). The criticism to the effect that the investigator often sets up in his own mind an arbitrary concept of what the Golgi apparatus should look like and then impregnates the material until the apparatus approaches his preconceived idea is in many cases

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still applicable. Unless a unique position or configuration of the Golgi apparatus is produced by experimental procedures, or unless the curve of variability is shifted significantly by the experimental situation, conclusions are very apt to be the result of subjective impressions of the investigator, and hence often difficult of verification by others. The fact that treatment of thyroid tissue in the Warburg respirometer at 38° C. appears to introduce no appreciable distortions of the Golgi material should provide a starting point for intensive study of this conspicuous organoid.

Of the various techniques which have been introduced for estimating cell height, the cell height index of Rawson and Starr (1938) has come into fairly common use. As the method rests on a subjective estimate of what constitutes an "average" cell height, difficulties are often encountered in the case of follicles whose opposite sides are lined with epithelium of greatly differing height or in follicles in which the cell heights are very unequal. By photographing sections of thyroid and then measuring the follicular walls with a planimeter certain obvious advantages are introduced. The subjective selection of a representative cell is eliminated, readings are obtained at once in terms of area, the proportion of cellular area to lumen area in the follicles is quickly calculated, and tables of constants are available by which the area readings are easily converted to volume. In this way total colloid may be fairly accurately determined. By use of proper sampling methods, the photographic-planimetric technique should consume little more time than a careful estimate by the cell height index method.

Perhaps the most striking finding in the present investigation concerns the increase in cell height caused by the *in vitro* exposure of the thyroid gland to cold. It has long been known that cold acting on the intact animal caused an increase in height of the follicular cells (Cramer and Ludford, 1926; Krogh, Lindberg and Okkels, 1932; Kenyon, 1933; Fischborn, 1935; Baillif, 1937; Starr and Roskelly, 1940; Ariel and Warren, 1943). This effect has been interpreted, however, as a stimulus to the anterior pituitary causing an increased production of thyrotropic hormone, since the thyroid stimulation after exposure to cold is abolished by hypophysectomy or section of the pituitary stalk (Uotila, 1939; Dempsey and Uotila, 1940). Since the observations made in this study show that cold acting on the isolated thyroid gland causes an increase in cell height, the question of the mechanism of the effect is raised anew. It may be accounted for as a direct temperature effect on thyroid cells or by assuming that the effect of thyrotropic hormone on thyroid cells is potentiated by cold, an effect similar to that found for the action of epinephrine on smooth muscle (Fuhrman, Crismon, Fuhrman and Field, 1944). Very small quantities of thyrotropic hormone might be carried into the Warburg vessels with the tissue slices (Cf. Eitel, Krebs and Loeser, 1933). An

alternative explanation for such difference in cell height would postulate that the histology of a single lobe of the thyroid gland is variable to this degree. In the light of the large number of studies on the histology of the thyroid gland, this interpretation seems improbable. Even moderate degrees of variability in the thyroid would nullify many previous cytological investigations of the gland, especially those observations based on litter-mate controls. In the present investigation the difference in cell height is most likely attributable to the effect of temperature, since there was no other variable and since portions of the same lobe of the same gland were used in the vessels at 5° C. and 40° C. That such increase in cell height should occur within an hour is not remarkable in view of the recent report by Ariel and Warren (1943) of increases in cell height within one-half hour *in vivo* as a result of cold. The appearance of the thyroid tissue run at 40° C. may be similarly interpreted in the light of investigations of Wagschal (1931), Okkels (1932, 1934), Severinghaus (1933), Gillman (1934) and others, to indicate a decrease in glandular activity as a result of the higher temperature. The anatomical location of the thyroid in the neck and against the trachea make it easily accessible to changes in environmental and air temperature. Such an arrangement may well be regarded merely as an added factor of safety in the regulation of body temperature.

SUMMARY

The oxygen consumption of thyroid tissue from guinea pigs was measured by the Warburg technique at 5° C., 38° C., and 40° C. These tissues were then examined histologically and compared with control portions of the same lobes of the same glands.

In those tissues with a high QO_2 due to temperature follicular cell height was relatively low. Conversely, low QO_2 was usually associated with an increased cell height. This change, known to occur likewise *in vivo*, apparently may be a direct response to temperature rather than an indirect effect of thyrotropic hormone. Tissues run at 38° C. showed no essential differences from their controls.

An improved method for direct determination of cell area and colloid area is suggested. The application of this method to the computation of cellular and colloid volume and hence to calculation of oxygen consumption of thyroid tissue under experimental conditions is discussed.

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ACTION OF ESTROGEN ON PLASMA CALCIUM AND ENDOSTEAL BONE FORMATION IN PARATHYROIDECTOMIZED PIGEONS

OSCAR RIDDLE, VITA M. RAUCH
AND GUINEVERE C. SMITH

From the Carnegie Institution of Washington, Department of Genetics
COLD SPRING HARBOR, NEW YORK

IT IS WIDELY RECOGNIZED that the parathyroid glands influence some of the processes concerned in the development and resorption of normal bone (Silberberg and Silberberg, 1943; McLean, 1943); and an effect of parathyroid extract on callus formation in the humerus of pigeons has been reported (Roche and Mourgue, 1939). The formation of endosteal bone following prolonged treatment with estrogens was first observed in fowl by Zondek (1937). Pfeiffer and Gardner (1938) and Bloom, McLean and Bloom (1941, 1942) have described in detail the histology of endosteal bone formation and resorption in pigeons. The literature on this aspect of the subject has been reviewed by Campbell and Turner (1942), McLean (1943), and Gardner and Pfeiffer (1943).

It is also well known that the parathyroids share in the regulation of the calcium level of the blood. Clearly, however, ovaries and estrogens participate in the hormonal regulation of the blood calcium of females of non-mammalian vertebrates. This is demonstrated by the following basic observations: (1) the ripening of eggs in the ovaries of pigeons is associated with a phenomenal increase in the blood calcium (Riddle and Reinhart, 1926); (2) at the same stage of reproduction a similar calcemia occurs in fishes, amphibia and other birds (many observers); (3) only or mainly the non-diffusible fraction of the blood calcium is increased in these calcemias of reproduction (Correll and Hughes, 1933; Laskowski, 1933); (4) similar and even greater increases in total blood calcium of birds result from the administration of estrogens, but not of androgens (Riddle and Dotti, 1936); (5) only or mainly non-diffusible calcium is increased in estrogen-induced calcemias (Riddle and McDonald, 1945). This literature was reviewed by Riddle (1942) and Gardner and Pfeiffer (1943).

The purpose of the present study was to learn whether the parathyroids are essential to either the calcemia or to the formation of

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endosteal bone which follows the administration of estrogen in pigeons. A preliminary report on these results has been published (Riddle and Rauch, 1944). After this study was completed it was learned that Benoit and associates (1941) reported that the calcemic action of estrogen is prevented by parathyroidectomy in ducks.

MATERIALS AND METHODS

Parathyroidectomy is performed with less difficulty in a large pigeon than in a small one. Since information on the location and organization of the parathyroid tissues of domestic pigeons was not found in the literature this point was carefully studied in a large breed, the White Carneau. In a dozen old and young specimens of this breed no trace of parathyroid tissue could be found within the thyroids. The two pairs of parathyroids lie on the jugulars 5 to 12 mm. posterior to the thyroids; thus they are usually separated by a still greater distance from the nearest normal lobe of thymus tissue. In these pigeons parathyroidectomy involved no injury whatever to the thyroids. It was found advantageous to remove the pair of glands on the right and left sides of the body in two operations separated by an interval of approximately one week. Just preceding the second operation the bird was given an injection of 0.1 ml. dihydrotachysterol (AT10). In many cases this dosage was repeated daily, together with oral administration of 0.2 gm. calcium in the form of gluconate, in order to prevent early and fatal tetanic convulsions; in other cases the post-operative treatment was limited to the feeding, by capsule, of 1.0 gm. daily of aluminum hydroxide (gelatinous) to restrict or prevent absorption of phosphorus from the intestine. The latter procedure maintained all operated birds in excellent condition, both those used as control and those which were estrogen-treated. At autopsy the region of the parathyroids was carefully searched for residual parathyroid tissue and any suspected tissue was sectioned. Postmortem study showed that some parathyroid tissue was present in about one-eighth of the operated birds; such birds are eliminated from this report. Treatment of hypophysectomized birds was started 24 hours after operation. The birds were fed a mixed grain diet and a commercial grit containing oyster shell, calcite, sand, salt and charcoal.

Since adult or maturing females may form endosteal bone without treatment (Kyes and Potter, 1934) only adult males and immature males and females were usable in the present study. Data for four adult females used in our initial tests are not included in this report. Study of the bones, principally the femurs, was limited to macroscopic observation. Most of the calcium data were obtained with the Koch and Hanke (1943) modification of the method of Roe and Kahn (1929) except that all of the trichloracetic acid supernatant was used (2.5 ml., obtained by careful drainage of the centrifuge tubes for 5 minutes) instead of 1 ml., the volume of the phosphate precipitating reagents being increased accordingly. The results are approximately 10 per cent too low since some of the supernatant always remained in the trichloracetic acid precipitate. Other calcium determinations were made by methods described by Riddle and McDonald (1945).

Crystalline estrogens and androstenedione were obtained from the Schering Corporation, through Dr. Erwin Schwenk. Diethylstilbestrol (Synestrol) was contributed by Sharp and Dohme. Estradiol benzoate was dis-

solved in an amount of fluid which varied from 0.1 to 0.3 ml. These variations in addition to the two quantities of hormone used, account for the exceptionally low and high values obtained for plasma calcium in certain tests. The dosage of control and operated birds subjected to the same test was identical, however, in all respects. Blood for calcium determinations was taken from parathyroidectomized ("operated") birds at 24 or more hours after their second operation. Only when one such sample had shown a marked reduction of plasma calcium, indicating completeness of the operation, was treatment started.

RESULTS

The results are presented in table 1. A group of 4 normal birds

TABLE 1. PLASMA CALCIUM AND OSSIFICATION OF FEMURS IN NORMAL AND PARATHYROIDECTOMIZED (OPERATED) PIGEONS INJECTED DAILY WITH ESTRADIOL BENZOATE (OR GIVEN OTHER TREATMENT, AS NOTED)

Normal or operated	Age	Number of birds	Plasma calcium		Grades of ossification				
			Before treatment	End of treatment	0	1	2	3	4
	mos.		mg. %	mg. %					
			4-day treatment, 0.5 mg.						
♂ ♀ Normal	2–4	16	8.9	26.0	13	3			
♂ ♀ Operated	2–4	25	6.0	20.6	20	5			
♂ ♀ Hypophysec.	2.2	4	9.1	19.1	4				
♂ Operated	10–60	6	6.1	20.1	4	2			
			8-day treatment, 0.25 mg.						
♂ ♀ Normal	2.2	3	11.4	15.1		3			
			14-day treatment, 0.5 mg.						
♂ ♀ Normal	1.4	4	9.4	24.2	1	2			1
♂ ♀ Hypophysec.	2.2	2		24.7				2	
			15-day treatment, 0.25 mg.						
♂ Normal	2–7	8[1]	9.2	23.5[1]		2	4	1	1[1]
♂ ♀ Operated	2–7	8	6.0	19.7			2	4	2
♂ Normal	5–30	4	9.4	20.3		1		2	1
♂ Operated	5	4	6.3	18.8			3	1	
			19–30-day treatment, 0.25 mg.						
♂ Normal	7–20	4	9.3	20.6			1	1	2
♂ Operated	5	2	5.6	14.5			1	1	
			15-day treatment, 0.5 mg. + $Al(OH)_3$						
♂ ♀ Normal	2.2	5[2]	11.0	36.6				4	1
♂ ♀ Operated[3]	2–4	3	4.6	52.3				1	2
			15-day sesame oil control, $Al(OH)_3$ only						
♂ ♀ Normal[4]	2.2	3	11.0	10.3	3				
♂ ♀ Operated[3]	2–4	3	4.4	9.4	3				
			14-day control on 0.4 ml. AT10						
♂ Normal	10	4	9.4	11.5	4				
			15-day control on 0.1 ml. AT10 + 0.2 gm. Ca						
♂ ♀ Normal	2.2	8	11.0	10.4	6	2			
			15-day androstenedione, 3.0 mg.						
♂ ♀ Normal	2.2	4	10.5	10.2	2	2			

[1] Three birds of this group were injected with diethylstilbestrol 0.1–0.3 mg. daily.
[2] Two of this group of 5 birds were fasted throughout the 15-day period.
[3] After operation these birds were given neither AT10 nor calcium gluconate.
[4] Not injected with sesame oil.

treated with 3 mg. daily of an androgen, androstenedione, showed no increase of plasma calcium. Averages obtained from entirely comparable tests indicate that treatment with estrogen increased the

plasma calcium of 36 normal birds by 16.3 mg. per cent, and that of 48 operated birds by 16.1 mg. per cent. It is notable, however, that the AT10 and calcium gluconate administered to many of the operated birds was probably responsible for a small part (2.0 mg. per cent) of the increase shown by them (table 2). The operated birds, however, lost weight and therefore probably consumed less food than the normal birds. The plasma calcium of the few operated birds maintained with $Al(OH)_3$, although initially quite low, reached almost normal levels after 20 days. These data warrant the conclusion that estrogen increases the plasma calcium by approximately the same amount in normal and parathyroidectomized pigeons.

Examination of the data concerning ossification of the femur (table 1) shows that individual birds, whether normal or operated,

TABLE 2. EXTENT TO WHICH SUBSTANCES ADMINISTERED DAILY TO MAINTAIN LIFE IN OPERATED BIRDS INCREASED THE PLASMA CALCIUM IN NORMAL AND PARATHYROIDECTOMIZED PIGEONS

Normal or operated	Age	Number of birds	Plasma calcium in mg. per cent					
			Control	At days				
				4	7	10	12	15	20
	mos.		AT10 (0.1 ml.) plus calcium gluconate (0.2 gm. Ca)						
♂ Normal	10.0	4	10.4		11.4			11.4	
♂ Normal	2.2	8	11.0	11.1[1]			10.8[2]	10.4	
♂ Operated	2.5	5	6.5		7.3	8.6			
♂ ♀ Operated	2.3	4	6.0	8.1					
			Aluminum hydroxide (gel); 1.0 gm.						
♂ ♀ Normal	2.2	3	11.0				10.4		10.3
♂ ♀ Operated	2–4	3	4.4	7.7	9.1		9.9		9.4

[1] Only 3 birds measured.
[2] Only 5 birds measured.

vary markedly in the amount of medullary bone formed in a unit of time under daily injections of estradiol benzoate. It is also evident that the presence or absence of the parathyroid glands did not measurably affect the amount of medullary bone formed in the femurs of these birds. The slowness of the process of ossification in these tests is especially notable. The daily injection of 0.5 mg. estradiol benzoate for 4 days induced even the lowest grade of ossification in only 20 per cent of 41 tests; one-half of that dosage over 8 days gave only grade 1 ossification in all of 3 tests. A minimum of 15 days under high dosage (0.5 mg. daily) was required to produce in all birds ossification of grades 3 or 4). The results suggest that femur ossification proceeds more rapidly in older birds, particularly in adult males aged 7 to 60 months, than in younger birds.

The effects of diethylstilbestrol on the formation of endosteal bone were entirely similar to those obtained with estradiol benzoate. When the androgen, androstenedione, was administered in huge doses

(3.0 mg. daily) over 15 days it showed no trace of effect on bone in two cases and a minimum (grade 1) effect in two cases. It seems probable that this minimum effect in some animals was due to the estrogenic potency of 45 mg. of this particular androgen. Parkes and Deanesly (1937) found that the estrogenic potency of androstenedione is nearly five times that of testosterone.

No earlier tests of the ability of hypophysectomized birds to form endosteal bone have been reported. Two such birds showed grade 3 ossification after treatment for 14 days; 4 birds were unaffected by treatment for 4 days. These tests indicate that removal of the anterior pituitary gland alone did not measurably modify this action of estrogen. Both pituitary and parathyroids were removed from 9 birds, but in such birds life was not maintained sufficiently long for adequate tests of the ability of estrogens to form bone.

One parathyroidectomized bird was maintained for 108 days during which it was subjected to four separate 4-day treatments with estradiol benzoate (0.5 mg. daily). The plasma calcium values at the end of these four treatments were, 21.8, 25.2, 20.0 and 20.3 mg. per cent. The two last-named values were obtained after an additional operation involving complete removal of the anterior pituitary gland. This bird exhibited tetany on two occasions 41 days apart; on the first occasion the total plasma calcium was very near to an extreme high (on the following day it was 25.2 mg. per cent), and the second attack coincided with the lowest value (5.6 mg. per cent) obtained from 19 measurements made on this bird. No tetany was observed during 21 days following removal of the hypophysis. When killed at the end of a final 4-day treatment the femur showed no formation of endosteal bone.

DISCUSSION

Several parathyroidectomized birds died either before or after treatment was started. Many others showed signs of tetany at one or more times during treatment when total blood calcium was certainly at higher than normal levels. Data relating to the partition of the calcium and phosphorus fractions in the blood of the birds used in this study are presented in another communication (Riddle and McDonald, 1945).

The slowness of the endosteal reactions under estrogen treatment, in comparison with that in the preovulating pigeon, was noted by Pfeiffer and Gardner (1938) and by Bloom, McLean and Bloom (1942). Those investigators used lower levels of dosage than was used in the present tests; and, although those earlier studies were restricted to adult males, ossification was less rapid and less complete than in the present tests. It therefore seems evident that in pigeons the degree of hyperossification and the time of its onset depend upon the size of the dose and on the length of the period of its administration. This relationship was earlier demonstrated in English sparrows by Pfeiffer,

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Kirschbaum and Gardner (1940) who also noted that relatively much more estrogen is required to form endosteal bone in sparrows than in pigeons. Landauer and Zondek (1944) noted this species difference in drakes. They reported also that effects are similar in mature cocks and fully grown capons, but that young cockerels show a much greater response than mature cocks. Our data suggest that adult male pigeons are more responsive than young males.

The present data were obtained at all seasons. Essentially similar grades of femur ossification were obtained in both young and adult males during November to January as in other parts of the year. Bloom, McLean and Bloom (1942) utilized doses which we would regard as very small and observed that "doses of α-estradiol sufficient to produce new bone in the medullary cavity of male pigeons during the breeding season are ineffective in male pigeons during the resting season (November-December), and in castrate males at any time." The fact that the daily addition of 0.5 to 2.0 mg. testosterone propionate rendered the daily dosage of estradiol (66.5 gammas) effective led these observers to conclude that the presence of androgen is necessary to the formation of endosteal bone. That conclusion is probably in error since, with higher doses of estrogen alone we were usually successful; one of 5 adult males treated in November-December died under injection of estrogen (0.25 mg. daily) for 14 days and it alone of the group examined on the following day had formed no medullary bone. It is possible that the estrogenic potency of the androgen used by Bloom, McLean and Bloom was sufficient, when added to that of a sub-threshold dose of estrogen, to exceed the threshold. Deanesly and Parkes (1937) observed that the androgen, *trans*-androstenediol, is about 1/2000 as active as estrone in assays made on the uteri of immature rabbits; testosterone propionate may have an even higher estrogenic activity, relative to α-estradiol, when assays are made on the basis of endosteal bone formation. The conditions under which the male pigeons were kept in the two laboratories may be partly responsible for the different results obtained, and certainly both laboratories observed that individual birds responded very unequally even in summer. Bloom, McLean and Bloom state that their birds were kept under "uniform conditions." In that case, however, it is difficult to understand why their birds attained a "resting season." Most pigeons provided with some heat during autumn and winter breed throughout the year.

Wartime conditions have prevented us from seeing copies of publications by Benoit and associates (1941) in which, apparently, it is stated that estradiol dipropionate did not increase the plasma calcium of parathyroidectomized ducks. These ducks, however, seem to have survived the operation by only one day.

SUMMARY

The four parathyroid glands of Carneau pigeons are entirely ex-

ternal and posterior to the thyroids. Apparently complete parathyroidectomy can be performed without injury to the thyroids.

Parathyroidectomized birds were maintained for long-term tests either by dihydrotachysterol and calcium gluconate or by feeding 1 gm. daily of aluminum hydroxide (gelatinous). The first-named procedure increased the plasma calcium by about 2.0 mg. per cent, and the latter by double that amount.

Daily doses of 0.25 to 0.5 mg. estradiol benzoate over 4 to 15 days increased the plasma calcium of 47 parathyroidectomized birds by 16.1 (−2.0) mg. per cent. Comparable treatment increased the plasma calcium of 36 normal pigeons by 16.3 mg. per cent.

Estradiol benzoate was approximately as effective in promoting the formation of endosteal (medullary) bone in the femurs of parathyroidectomized pigeons as in normal pigeons. It was also effective in hypophysectomized immature pigeons.

Adequate doses of estradiol benzoate promoted the formation of endosteal bone of male pigeons during all seasons of the year. In doses of 3 mg. daily for 15 days the androgen, androstenedione, gave macroscopic evidence of minimum endosteal bone formation in two tests and of no effect in two tests. The effect, when present, probably resulted from the estrogenic potency of this androgen.

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THE PARTITION OF PLASMA CALCIUM AND INORGANIC PHOSPHORUS IN ESTROGEN-TREATED NORMAL AND PARATHYROIDECTOMIZED PIGEONS

OSCAR RIDDLE AND MARGARET R. McDONALD
From the Carnegie Institution of Washington, Department of Genetics,
COLD SPRING HARBOR, NEW YORK

IN OVIPAROUS VERTEBRATES each period of egg production is associated with a large increase in the non-ultrafiltrable fraction of the plasma calcium. Similar and even greater increases in total plasma calcium of these vertebrates result from the administration of estrogens, but in these cases the partition of calcium has not been determined. The literature on this subject has been reviewed by Riddle (1942) and by Gardner and Pfeiffer (1943). A recent study by Riddle, Rauch and Smith (1945) has shown that parathyroidectomy has little or no effect on the ability of estrogens to cause hypercalcemia and medullary bone formation in the pigeon. Many of the same animals used in that investigation were further utilized for the present study of the partition of calcium and inorganic phosphorus in the plasmas of estrogen-treated pigeons.

Grollman (1927) showed that although the inorganic phosphorus of mammalian blood is normally completely ultrafiltrable this is only partially true for some lower vertebrates; moreover, in those lower vertebrates the blood calcium concentration was higher than that usually found in mammals. Various workers have shown that the non-ultrafiltrable inorganic phosphorus of the plasma is a colloidal form of calcium phosphate. Schmidt and Greenberg (1935) and Greenberg (1939) have reviewed the literature on the formation and nature of this colloid, and Greenberg and Larson (1939) have postulated that its composition is $Ca_3(PO_4)_2$.

The inorganic phosphorus content of the blood plasma of the ovulating hen was shown by Laskowski (1933; 1934) and by Greenberg and associates (1936) to be greater than that of the non-ovulating hen; according to these studies the increase occurred in the non-ultrafiltrable fraction. On the other hand, Benjamin and Hess (1933), Heller, Paul, and Thompson (1934), and Roepke and Hughes (1935) observed no increase in the inorganic phosphorus of fowl serum due to egg-laying. Laskowski (1936) found that the total inorganic phosphorus of the plasma of various birds, reptiles, and fishes was increased during the egg-laying period. However, Schrire (1942) re-

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ported a fall in the serum inorganic phosphorus of the female toad, *Xenopus*, during the breeding season, and also observed (1942a) a marked decrease in the same constituent following injections of progesterone and of estradiol benzoate into normal, hypophysectomized and gonadectomized toads. According to Marlow and Koch (1937) small doses of estrogenic extracts did not alter the inorganic phosphorus of fowl serum. Landauer and associates (1941) noted that larger doses of estrogens increased the total inorganic serum phosphorus of ducks. According to Bloom, McLean and Bloom (1942) 1 to 50 gammas of estradiol daily for 14 days had little if any action on the serum phosphate of pigeons.

The results presented here show that larger doses of estrogens markedly increase the plasma calcium and inorganic phosphorus of normal, parathyroidectomized and hypophysectomized pigeons; that, in the case of both of these minerals, the increase occurs in the non-ultrafiltrable fraction; and that there is a direct relationship between the increase in these two components.

EXPERIMENTAL METHODS

The birds used and their treatment have been described elsewhere (Riddle, Rauch and Smith, 1945). Twenty-nine additional normal birds which received no treatment whatsoever were included in the present study. Blood was drawn from a wing vein, using heparin as anticoagulant, centrifuged immediately, and the plasma analyzed as follows:

Ultrafiltration. Ultrafiltration was performed by the method of Greenberg and Gunther (1930) with substitution of viscose membranes for collodion sacs.

Phosphorus. Both total and ultrafiltrable inorganic phosphorus were determined according to King (1932).

Calcium. Total and ultrafiltrable calcium were precipitated as calcium phosphate by the method of Roe and Kahn (1929), the precipitate washed by the same method, and the calcium phosphate analyzed according to King (1932). The non-ultrafiltrable values, in the case of both minerals, were obtained as differences between the total and ultrafiltrable values.

RESULTS

The effect of estrogens on the ultrafiltrable and non-ultrafiltrable plasma calcium and inorganic phosphorus in normal, parathyroidectomized and hypophysectomized pigeons are summarized in table 1. The treatment of the various groups of birds, including the substances used to maintain life in birds deprived of their parathyroids, is indicated. Birds of the latter type were fed calcium (200 mg. daily) in the form of calcium gluconate, and injected daily (or less frequently) with 0.1 ml. of commercial dihydrotachysterol (AT10). Some parathyroidectomized birds were maintained with aluminum hydroxide (gelatinous).

The tabulated data show that parathyroidectomy in pigeons, as in mammals, decreases the ultrafiltrable plasma calcium. Treatment with calcium gluconate and dihydrotachysterol alleviates this decrease, while continued therapy with alumina gel practically elimi-

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nates it. Estrogens induced no significant change in the ultrafiltrable plasma calcium of either normal, parathyroidectomized, or hypophysectomized pigeons. All of the observed rise in plasma calcium

TABLE 1. THE EFFECT OF ESTROGEN, ANDROGEN AND VARIOUS CONTROL TREATMENTS ON THE PARTITION OF CALCIUM AND INORGANIC PHOSPHORUS IN NORMAL, PARATHYROIDECTOMIZED AND HYPOPHYSECTOMIZED PIGEONS

Treatment (daily)			Age at killing	Number of birds	Milligrams per 100 ml. plasma			
					Calcium		Inorganic P	
Substance	Dosage	Duration			Ultrafiltrable	Non-ultrafiltrable	Ultrafiltrable	Non-ultrafiltrable
	mg.	days	mos.					
Normal								
None			1.8	5	5.7	5.1	4.8	1.3
None			2.7	13	6.2	4.2	4.3	0.9
$Al(OH)_3$	1000	19	2.9	3	6.5	3.8	4.2	0.4
AT10 (0.1 ml.) +Ca	200	15	2.8	8	6.7	3.7	3.9	0.6
Estradiol benzoate	0.5	5	2.5	4	7.4	28.4	3.8	5.2
Estradiol benzoate	0.25	8	2.5	3	7.6	7.5	4.0	1.4
Estradiol benzoate	0.5	14	1.8	4	5.2	19.0	4.8	3.4
Estradiol benzoate	0.5	15	2.8	5	6.3	30.4	2.9	6.4
Androstenedione	3.0	15	2.7	3	6.8	3.4	4.5	0.3
None			Adult	11	6.6	4.0	2.4	0.7
Diethylstilbestrol	0.2	15	Adult	3	6.7	14.8	4.0	2.0
Estradiol benzoate	0.25	25	Adult	2	6.7	21.2	4.1	3.6
Parathyroidectomized								
AT10 (0.1 ml.) +Ca	200	9	2.3	4	4.5	3.5	9.9	0.1
$Al(OH)_3$	1000	20	3.2	3	6.5	2.9	6.3	0.5
Estr. b. (+$Al(OH)_3$)	0.5	15	3.3	3	6.9	45.4	4.2	8.6
Estr. b. (+AT10+Ca)	0.25	15	2.5	2	1.5[1]	11.4[1]	11.4[1]	0.7[1]
Estr. b. (+AT10+Ca)	0.25	15	2.5	2	4.2	17.8	4.2	3.8
AT10 (0.1 ml.) +Ca	200	14	Adult	5	5.1	4.6	6.9	0.7
AT10 (0.1 ml.) +Ca	200	5[2]	Adult	1	0.5[2]	5.2[2]	10.6[2]	0.1[2]
Estr. b. (+AT10+Ca)	0.25	15	Adult	3	5.1	17.6	5.7	3.3
Estr. b. (+AT10+Ca)	0.25	15	Adult	1	0.2[3]	19.7[3]	12.1[3]	0.0[3]
Hypophysectomized								
None		0[4]	2.7	3	5.5	3.6	4.7	1.0
Estradiol benzoate	0.5	3	2.8	3	6.1	13.0	3.8	2.7
Estradiol benzoate	0.5	14	2.7	2	7.0	17.6	4.2	3.5

[1] Both birds dying in tetany when sampled.
[2] Operated 180 days; treated intermittently. Dying in tetany when sampled.
[3] Bird dying in tetany.
[4] Blood samples taken 15 days after operation.

occurred in the non-ultrafiltrable fraction which showed as much as a 10-fold increase under the influence of estrogens.

The tabulated data show that the ultrafiltrable inorganic phosphorus of normal pigeons progressively decreases with increasing age. Similar results have been found by Greenberg *et al.* (1936) for the ultrafiltrable inorganic phosphorus of fowl serum, and by Stearns and Warweg (1933) for the total inorganic phosphorus of human serum. The data also show that the ultrafiltrable inorganic phosphorus of pigeon plasma is increased by parathyroidectomy. When these factors for age and operation are taken into account the data clearly demonstrate that the inorganic phosphorus content of pigeon plasma was increased by treatment with estrogens (but not with androgen). The increase occurs in the non-ultrafiltrable fraction and is of the same magnitude in normal, parathyroidectomized[1] and

[1] The only exceptions were birds dying in tetany when sampled.

and hypophysectomized pigeons. Estrogens induced no significant[2] change in the ultrafiltrable inorganic phosphorus.

The increase in the non-ultrafiltrable inorganic phosphorus induced by estrogen parallels the increase in non-ultrafiltrable calcium. When the individual data for these fractions are plotted against each other, as has been done in figure 1, it is apparent that there is a direct

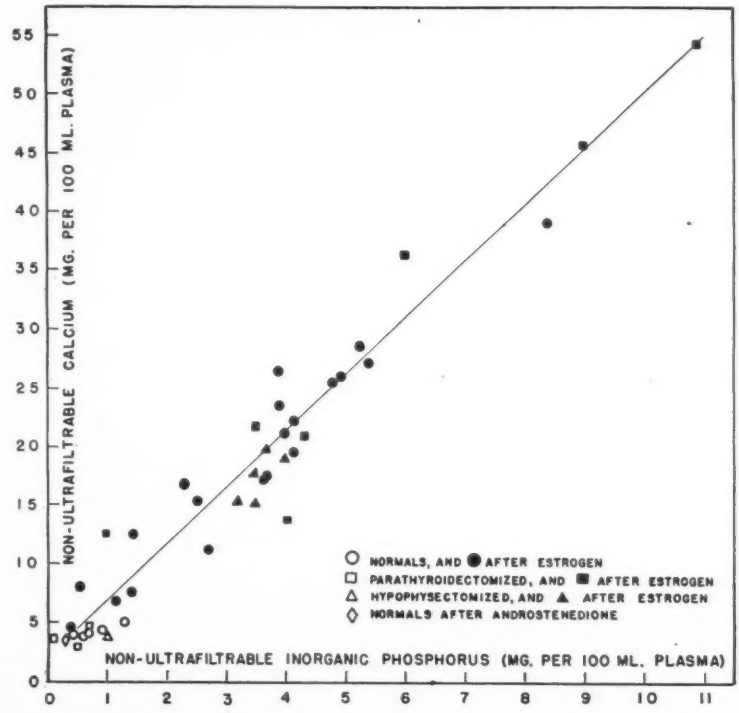

FIG. 1. Correlation diagram of non-ultrafiltrable calcium and the non-ultrafiltrable inorganic phosphorus of the blood plasma of pigeons variously treated with estrogen.

relation between the increases in non-ultrafiltrable calcium and inorganic phosphorus. When the correlation coefficient is calculated for the estrogen-treated pigeons we obtain the highly significant value, $\gamma = .960 \pm .014$. A similar relationship ($\gamma = .88$) was observed by Greenberg *et al.* (1936) in sera of laying fowl.

Greenberg and Larson (1939) have shown that the non-ultrafiltrable inorganic phosphorus probably exists in the form of colloidal $Ca_3(PO_4)_2$. Greenberg *et al.* (1936) earlier reported that in ovulating

[2] The small increase in the ultrafiltrable inorganic phosphorus of the plasma of the small group of normal, estrogen-treated, adult pigeons is probably not significant. Much unpublished data of the authors show that the increase in inorganic phosphorus during the normal reproductive cycle of the pigeon is all in the non-ultrafiltrable fraction.

fowl 7.5 of a total of 22.8 mg. per cent of non-diffusable calcium was in the form of colloidal calcium phosphate. Using the formula, $Ca_3(PO_4)_2$, calculations made from the data of table 1 show that 36.8 per cent of the non-ultrafiltrable calcium in estrogen-treated pigeons can be accounted for in this form. This value is in agreement with that obtained by Greenberg.

SUMMARY

Estrogens increase the plasma calcium and inorganic phosphorus of normal, parathyroidectomized, and hypophysectomized pigeons. The effect is primarily on the non-ultrafiltrable fraction of both of these components. The increases varied from 2 to 10 fold and were approximately equal in the three types of birds. No significant change was observed in the ultrafiltrable fraction of either calcium or phosphorus.

In estrogen-treated pigeons there is a direct relationship between the increase in the non-ultrafiltrable fractions of calcium and inorganic phosphorus. A correlation coefficient of $.960 \pm .014$ was obtained. One-third of the non-ultrafiltrable calcium in such pigeons can be accounted for as colloidal calcium phosphate.

In three tests on normal pigeons the androgen, androstenedione, did not increase either ultrafiltrable or non-ultrafiltrable calcium or phosphorus.

Parathyroidectomy in the pigeon is usually followed by a decrease in ultrafiltrable calcium and an increase in ultrafiltrable phosphorus. Prolonged administration of aluminum hydroxide (gel) reversed both of these changes.

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THYROTROPHIC HORMONE CONTENT OF THE BLOOD SERA AND PITUITARY GLANDS OF THIOUREA-, SULFADIAZINE-TREATED AND THYROIDECTOMIZED RATS[1]

ALBERT S. GORDON, E. D. GOLDSMITH
AND HARRY A. CHARIPPER

From the Department of Biology, Washington Square College of Arts and Science, New York University

NEW YORK, NEW YORK

THE ADMINISTRATION of thiourea, its derivatives and certain of the sulfonamides to mammals results in the development of an enlarged hyperplastic thyroid associated with a state of functional hypothyroidism (the Mackenzies, 1943; Astwood, Sullivan, Bissell and Tyslowitz, 1943). Experiments with radioactive iodine (Franklin and Chaikoff, 1943, 1944; Keston, Goldsmith, Gordon and Charipper, 1944; Schachner, Franklin and Chaikoff, 1944; Franklin, Chaikoff and Lerner, 1944; Rawson, Tannheimer and Peacock, 1944; Franklin, Lerner and Chaikoff, 1944) have shown that these drugs act by interfering with the production of normal thyroid principle. It is then postulated (the Mackenzies, 1943; Astwood, *et al.*, 1943) that the lowered thryoxine concentration of the blood, or the resulting lowered metabolic rate of the body, invokes an increased production of the thyrotrophic factor which, in turn, induces marked overgrowth and histological hyperactivity of the thyroid gland.

Although the studies performed with radioactive iodine are conclusive in demonstrating the mode of action of these drugs on thyroid metabolism, there are at present no physiological data available to verify the proposed behavior of the pituitary thyrotrophic mechanism under the influence of these anti-thryoid drugs. It is the purpose of this report to clarify further this mechanism by supplying information regarding the amounts of thyrotrophic hormone in the blood sera and hypophyses of rats treated with thiourea or sulfadiazine. For the sake of comparison similar data for surgically thyroidectomized rats will also be presented. A preliminary report on some phases of this work has already been published (Gordon, Goldsmith and Charipper, 1943b).

MATERIALS AND METHODS

Adult male rats weighing 185–250 gm. were used as serum and pituitary donors in all the experiments. In the first experiment, 30 rats were fed a

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[1] Aided by a grant from the Commonwealth Fund.

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normal laboratory ration to which was added 1.0% thiourea (Eimer and Amend or Eastman Kodak) or 1.0% sulfadiazine.[2] One group of 10 received the thiourea for 14 days and another group of 10, for 45 days. A third group of 10 rats was fed the sulfadiazine ration for 45 days. Twenty untreated rats served as the controls. At the end of the 14 and 45 day periods of treatment, all animals were anesthetized lightly with ether and the blood withdrawn by cardiac puncture. All blood from animals in the same group was pooled and allowed to clot in a refrigerator. Twenty-four hours later, the serum was pipetted off, centrifuged for 5 minutes at 3,000 R.P.M., and then passed through a Berkefeld filter.

The pooled pituitaries from the same groups of rats were reduced to a homogeneous pulp. The minced glands secured from each group of 10 rats were diluted with 100 cc. of distilled water. Prior to being used, 1 cc. of this suspension was further diluted with 9 cc. of distilled water. The sera and pituitary suspensions were stored in sterile flasks in the freezing compartment of a refrigerator.

The pooled blood and hypophyses from 10 thyroidectomized rats (6 months following thyroid removal) and another group of 10 control rats were subjected to similar procedures.

In another experiment, 10 rats were fed the thiourea diet for 20 days. They were then thyroidectomized and sacrificed 48 hours later. During this 48 hour period they continued to receive the thiourea ration. Ten additional rats, fed the normal laboratory ration, were also thyroidectomized and killed 48 hours later. The blood sera and pituitary suspensions were obtained in a manner similar to that described above.

All sera and glands were assayed for thyrotrophic potency in tadpoles according to the method of D'Angelo, Gordon and Charipper (1942). Upon attaining the 4 to 6 mm. hind limb stage, the tadpoles were injected pleuroperitoneally, once every 2 days, with 0.05 cc. of the blood sera or the pituitary suspensions. Control tadpoles received 0.05 cc. injections of distilled water. No food was given during the experimental treatment. The heads of at least 3 representative animals from each series were prepared for histological studies of the thyroid glands.

In a final experiment 24 larvae were injected once every 2 days with 0.04–0.05 mg. thiourea[3] in 0.05 cc. distilled water and measurements of hind limb length made and times of forelimb extrusion observed after 5, 11, 14 and 17 injections. Twenty-four larvae injected with 0.05 cc. distilled water served as controls. All animals in this series received food during the experimental period.

RESULTS

Effects of thiourea and sulfadiazine. The data of Table 1 disclose that the feeding of the two drugs for 45 days results in a significant lowering in the quantity of principle capable of inducing metamorphosis in both the blood sera and pituitary glands of rats. This is evidenced in the smaller rate of hind limb development and in the smaller

[2] We wish to thank Dr. John Henderson, Sharp and Dohme, Inc., for a generous supply of sulfadiazine.

[3] This is the maximal amount of the drug found to be present in 0.05 cc. of blood serum from rats fed 1.0% thiourea for 30 days (method of Williams, Jandorf and Kay, 1944).

number of forelimbs acquired by the tadpoles receiving sera and hypophyses from rats fed the drugs. The reduction in concentration of the principle is more marked with sulfadiazine than with thiourea. The mean thyroid and pituitary weights in mg. ± the average devi-

TABLE 1. RATE OF DEVELOPMENT OF RANA PIPIENS LARVAE INJECTED WITH BLOOD SERUM OR PITUITARY GLANDS FROM RATS TREATED WITH THIOUREA OR SULFADIAZINE FOR 45 DAYS

Materials injected	H.L.[1] increase (mm.) after 8 inject. ±S.E.[2]	No. FL[1] after 8 inject.
Norm. Ser.	6.3±0.21	2
Thio. Ser.	2.9±0.10	0
Sulfad. Ser.	2.1±0.19	0
Norm. Pits.	5.6±0.18	12
Thio. Pits.	3.5±0.14	1
Sulfad. Pits.	1.8±0.18	0

[1] H.L. and FL refer respectively to hind limb length and forelimb.
[2] Standard Error.
Twenty-five larvae were used to test each of the 6 materials.

TABLE 2. RATE OF DEVELOPMENT OF RANA PIPIENS LARVAE INJECTED WITH BLOOD SERUM OR PITUITARY GLANDS FROM RATS TREATED WITH THIOUREA FOR 14 DAYS

Materials injected	H.L.[1] increase (mm.) after 8 inject. ±S.E.[2]	No. FL[1] after 8 inject.
Norm. Ser.	5.7±0.22	0
Thio. Ser.	0.9±0.06	0
Norm. Pits.	4.3±0.20	3
Thio. Pits.	0.8±0.09	0

[1] H.L. and FL refer respectively to hind limb length and forelimb.
[2] Standard Error.
Twenty-five larvae were used to test each of the 4 materials.

ation respectively for the 3 series of donor rats were: thiourea-treated —44.5±7.5 and 7.7±0.7; sulfadiazine-treated—47.6±10.0 and 8.6 ±0.2; normal-untreated—20.5±2.0 and 7.4±0.4.

In order to determine whether similar effects could be obtained in a shorter period of time, a second experiment was performed in which the thiourea ration was fed for only 14 days. Examination of Table 2 indicates that here again a significant reduction is brought about by the drug in the quantity of substance capable of producing metamorphosis present in both the blood serum and pituitary glands. The mean thyroid and pituitary weights in mg. ± the average deviation respectively for the 2 groups of donor rats were: thiourea-treated— 28.5±5.5 and 6.4±0.4; normal-untreated 14.8±0.6 and 6.7±0.7.

The thyroid glands of the tadpoles injected with the serum from normal rats almost invariably appear more activated (i.e. possess higher epithelium and less colloid) than those of larvae given serum from the rats fed the drugs.

Effects of surgical thyroidectomy. That thyroidectomy increases the thyrotrophic hormone content of the blood and pituitary glands may be seen from the results in Table 3. The increase in the blood serum is

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slight but statistically significant. There can be no doubt, however, of the elevated level of this hormone in the pituitary. The hypophyses from the thyroidectomized rats averaged 8.4 ± 1.8 mg. as against 6.7 ± 0.4 mg. for the untreated controls.

Effects of thiourea followed by thyroidectomy. The reduced thyrotrophic hormone concentration in the blood of rats fed thiourea may be converted to an increased one by thyroidectomy as seen in Table 4. Following removal of the thyroid gland from rats given thiourea ration for 20 days, the thyrotrophin level of the blood is elevated after 48 hours and assumes a level above that in the controls. The values of

TABLE 3. RATE OF DEVELOPMENT OF RANA PIPIENS LARVAE INJECTED WITH BLOOD SERUM OR PITUITARY GLANDS FROM RATS THYROIDECTOMIZED FOR 6 MONTHS

Materials injected	H.L.[1] increase (mm.) after 8 inject. ±S.E.[2]	No. FL[1] after 8 inject.
Norm. Ser.	4.8±0.14	0
Thyrdx. Ser.	5.5±0.10	0
Norm. Pits.	4.7±0.13	0
Thyrdx. Pits.	6.9±0.27	6

[1] H.L. and FL refer respectively to hind limb length and forelimb.
[2] Standard Error.
Thirty larvae were used to test each of the 4 materials.

TABLE 4. RATE OF DEVELOPMENT OF RANA PIPIENS LARVAE INJECTED WITH BLOOD SERUM OR PITUITARY GLANDS FROM RATS TREATED WITH THIOUREA FOR 20 DAYS AND THEN THYROIDECTOMIZED

Materials injected	H.L.[1] increase (mm.) after 8 inject. ±S.E.[2]	No. FL[1] after 8 inject.
Norm. Ser.[3]	4.6±0.12	0
Thio-thyrdx. Ser.[4]	5.3±0.10	0
Norm. Pits.[3]	4.7±0.23	1
Thio-thyrdx. Pits.[4]	2.3±0.13	0

[1] H.L. and FL refer respectively to hind limb length and forelimb.
[2] Standard Error.
[3] From rats fed normal diet, thyroidectomized and sacrificed 48 yours later.
[4] From rats fed thiourea diet, thyroidectomized and sacrificed 48 hours later.
Thirty larvae were used to test each of the 4 materials.

the Standard Error indicate that this difference is significant. A comparison of the data for normal rat serum in the four tables will indicate that no change in the thyrotrophic potency of the blood in the normal rats occurs 48 hours after thyroidectomy. The thyrotrophin level in the hypophyses of rats fed normal ration still remains above that found in the thiourea-treated rats 48 hours after thryoid removal (Table 4).

Effects of small amounts of thiourea on amphibian development. Measurements of the larvae injected with 0.04–0.05 mg. thiourea reveal that these quantities exert no significant effect on the course of amphibian metamorphosis. Thus after 5, 11, 14 and 17 injections the hind limb lengths and numbers of animals acquiring forelimbs are

approximately the same as for the controls injected with distilled water.

DISCUSSION

The results obtained in the present report lend themselves to the following interpretation. The fact that thiourea, related compounds and the sulfonamides inhibit the formation of thyroid principle has already been well established (see Introduction for references). As hypothesized by the Mackenzies (1943) and Astwood *et al.* (1943), this reduced thyroxine concentration in the blood, or the resulting lowered metabolic rate, stimulates the anterior lobe of the hypophysis to release greater quantities of thyrotrophic hormone into the circulating blood. That the pituitary is actually concerned with this response is supported by the finding that hypophysectomy abolishes the reaction, and by the observation that "thyroidectomy-cells" appear in the anterior lobes of the drug-treated animals (the Mackenzies, 1943; Astwood *et al.*, 1943).

The fact that the blood serum of rats fed thiourea or sulfadiazine contains smaller amounts of thyroid-stimulating principle might be considered at variance with the view expressed above. An explanation of this apparent discrepancy, however, is indicated in the *in vitro* studies of Seidlin (1940) and Rawson, Sterne and Aub (1942). These workers have found that thyroid tissue is capable of removing thyrotrophic hormone from the solutions bathing it; in this respect it is much more effective than any other tissue. In addition, Loesser (1934) has shown that injected thyrotrophin disappears more quickly from the blood of a normal rabbit than from a thyroidectomized one and Seidlin (1940) has found that the urine of thyroidectomized guinea pigs injected with thyrotrophin possesses thyrotrophic activity whereas this hormone is absent in the urine of injected normal pigs. It is thus conceivable that the thyroid, in becoming so markedly enlarged and hyperplastic under the influence of the drugs, removes from the blood and utilizes considerable quantities of thyrotrophin.

Support for this explanation comes first from the results obtained with thyroidectomized rats. Here it is found that the thyrotrophic factor released from the hypophysis tends to accumulate excessively in the blood since, most likely, the end organ which normally removes it is not present. The hormone concentration is also elevated in the pituitary of the thyroidectomized animal probably because of its increased production and the absence of its utilization in the body. This phenomenon can be likened to the well known finding that an increase in the amount of gonadotrophin occurs in the hypophysis following castration (Engle, 1929) accompanied, presumably, by an increase in the amount released into the blood (DuShane, Levine, Pfeiffer and Witschi, 1935).

Additional evidence for the hypothesis is afforded by the experiment in which thiourea feeding is followed by thyroidectomy. In this

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case, 48 hours after thyroid removal from rats fed thiourea, the amount of thyrotrophin in the blood has not only increased but has actually exceeded the quantity detectable in the sera of the control rats thyroidectomized for the same period of time. This result cannot be attributed to any increase brought about by the effects of the operation *per se* since a study of the data will show that the hormone level remains unchanged in normal rats 48 hours after thyroidectomy. It indicates rather that the stimulated pituitary in the rat treated with thiourea continues to release relatively large quantities of thyrotrophin which, in the absence of the thyroid, accumulates in the blood. The 48 hour period following thyroidectomy was chosen arbitrarily. It is possible that if a different period had been employed, greater amounts of the hormone might have been found in the serum. The thyrotrophic potency of the hypophyses of the rats fed thiourea ration and thyroidectomized for 48 hours still remains smaller than that of animals given the normal diet. This may represent another manifestation of the continued augmented release of thyrotrophin set off by the drug action.

The observation that the thyrotrophic hormone content of the hypophysis is increased following surgical thyroidectomy is in agreement with the results reported by Chen and Van Dyke (1936) for the thyroidectomized female rabbit, and Zeckwer (1936) for the thyroidectomized rat but is at variance with the work of others (Houssay, Novelli and Sammartino, 1932; Kuschinsky, 1933; Hohlweg and Junkmann, 1933) who have found no alteration in thyrotrophic potency of the pituitary following thyroid removal. It is conceivable that the greater sensitivity of the tadpole test used for assaying thyrotrophic hormone in the current work makes it a superior method for distinguishing small differences in thyrotrophic hormone content. It is also to be observed that a slight hypertrophy of the hypophysis occurs in our rats 6 months after thyroid removal which is in agreement with most of the published work (see Van Dyke, 1936 and 1939). Because of this enlargement it is possible that the greater thyrotrophic potency of the hypophyses of these animals is not due to an augmented concentration but simply to an elevation in the total amount of hormone in the gland. Either of these conditions could account for the greater quantity of thyrotrophic principle found in the blood sera of surgically-thyroidectomized rats.

Also in agreement with our results is the finding that increased amounts of thyrotrophin are demonstrable in the blood following thyroidectomy (Emerson and Cutting, 1932) and in the blood sera or the urine of patients suffering from myxedema or other types of hypothyroidism (Fellinger, 1936; Hertz and Oastler, 1936, Rawson and Starr, 1938; Sendrail and Tamalet, 1939; Jones, 1939). Of especial significance and relevance to this report is the observation that the blood of patients afflicted with hyperthyroidism contains *reduced*

quantities of thyrotrophic principle (Fellinger, 1936; Bodart and Fellinger, 1936). In addition, subnormal quantities of thyrotrophin are reported in the urine of hyperthyroid human beings and animals (Smith and Moore, 1933; Hertz and Oastler, 1936; Antognetti and Geriola, 1936; Emerson and Cutting, 1938; Rawson and Starr, 1938; Starr, Rawson, Smalley, Doty and Patton, 1938; Sendrail and Tamalet, 1939; Jones, 1939). The situation is summarized in a conclusion made by Means (1939) that the blood and urine of myxedematous people or patients who have undergone thyroidectomy contain more, and the blood and urine of thyrotoxic patients possess less thyrotrophic principle than that found in the corresponding fluids of normal people. The suggestion has been made in the present work that the thyroid gland, in enlarging under the influence of thiourea and related substances, removes relatively large quantities of thyrotrophin from the circulation. It is possible as Rawson, Sterne and Aub (1942) have also suggested that a similar situation prevails in patients afflicted with Graves' disease, thus accounting for the smaller quantities of the hormone present in their body fluids. Increased metabolism of the thyrotrophic principle in hyperthyroidism also affords a possible explanation for this phenomenon but this could not be the case in thiourea-treated animals and humans where there is a marked reduction in metabolic rate (the Mackenzies, 1943; Astwood *et al.*, 1943; Astwood, 1943).

It has been pointed out (Williams, Weinglass, Bissell and Peters, 1944) that thiouracil, administered orally to rats, tends to accumulate in the blood up to 12 mg. per cent. It is also known that thiourea and thiouracil inhibit amphibian metamorphsis and the effect of simultaneously administered thyrotrophin on tadpole development (Gordon, Goldsmith and Charipper, 1943a; Hughes and Astwood, 1944). The possibility therefore exists that the reduced metamorphosis-stimulating capacity of the sera from rats fed thiourea is due in part to the presence of the drug in the blood. The data of the final experiment under Results show, however, that the quantity of thiourea present in the blood of rats fed the drug is not of sufficient magnitude to interfere with the developmental processes of amphibian larvae. Similarly, it is not possible to attribute the reduced activity of serum obtained from rats fed sulfadiazine to the presence of the drug in their blood since we have found (Gordon, Goldsmith and Charipper, in press) that sulfadiazine produces no inhibitory influence on amphibian development.

In a series of recently conducted metabolic studies (Gordon, Goldsmith and Charipper, in preparation) it has been found that thiourea exerts a considerable diuretic effect when fed to rats. This might result in an increased elimination of thyrotrophin and thus also account for the reduced quantities of the hormone in the blood sera of thiourea-fed rats. It is regrettable that no assays for urinary thyro-

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trophin were made in this study but the possibility of the increased excretion of this hormone appears to be precluded by the experiment in which thiourea treatment is followed by thyroidectomy (Table 4). In this case, although thiourea administration is continued during the 48 hours following thyroid removal, the thyrotrophic hormone in the blood, as has already been pointed out, does not remain at its low level but actually rises above that in the control. The same experiment also renders unlikely the possibility of any direct combination between thiourea and thyrotrophin to form an inert moiety.

Examination of the data in the Tables indicates some variation in the rate of hind limb growth in the 4 groups of tadpoles injected with serum from normal rats. This can be ascribed partly to seasonal variations in the hormone levels in blood sera of the rats since the four experiments were conducted at different times of the year. Other factors involved are probably different sensitivities of different batches of tadpoles and slight differences in laboratory temperature at the times the experiments were conducted. Comparisons are therefore reliable only when made between the groups of animals run simultaneously.

It should be emphasized that histological studies of the thyroids of the tadpoles used for assaying the blood sera from all the groups of rats indicate that the differences in effect on metamorphosis are not due to thyroid hormone but rather to differences in the amounts of thyroid-stimulating principle. Rat serum is known to contain relatively large quantities of thyrotrophin (van Caulaert, Aron and Stahl, 1931; Aron, 1931). That the ability of rat serum to stimulate metamorphosis is due largely to its thyrotrophin content has already been reported (D'Angelo, Gordon and Charipper, 1943; Gordon, Tornetta, D'Angelo and Charipper, 1943).

Thiourea and related substances have been utilized within the last year with considerable success in further elucidating the subject of thyroid chemistry and physiology. The present report indicates that it may also be used as an agent in shedding additional light on the thyrotrophic hormone production, release and utilization mechanisms within the body.

SUMMARY

The feeding of thiourea or sulfadiazine to rats for 14 or 45 days causes a diminution in the quantity of thyroid-stimulating factor in the blood sera and pituitary glands as judged by the effects of such materials on the rate of metamorphosis of Rana pipiens larvae.

Rats thyroidectomized for 6 months possess increased amounts of thyrotrophic hormone in their blood sera and hypophyses.

Rats fed thiourea for 20 days and then thyroidectomized possess increased quantities of thyrotrophin in their blood 48 hours after the operation; the concentration of this hormone in the pituitary glands of such animals is below that found in the glands of rats fed a normal diet and thyroidectomized for the same period of time.

The results are best interpreted on the basis that thiourea and sulfadiazine, by depressing the formation of active thyroid principle, cause an increased release of thyrotrophin from the pituitary into the blood where, however, it appears in reduced amount because of its removal and increased utilization by the enlarging thyroid gland.

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NOTES AND COMMENTS

THE EFFECT OF ADRENALECTOMY ON ALLOXAN DIABETES

ALLOXAN DIABETES is similar in many respects to diabetes mellitus. Several investigators, including Dunn, Kirkpatric, McLetchie and Telfer (1943), Bailey and Bailey (1943) and Goldner and Gomori (1943) have described the degeneration of the pancreatic islands in a variety of experimental animals following the administration of moderate doses of alloxan. Severe hyperglycemia, glycosuria and other diabetic symptoms, similar to those seen in diabetes mellitus, have been observed in these animals. Although the primary and important effect of alloxan is on the islet cells Goldner and Gomori (1943) have found renal damage and certain degenerative and inflammatory changes in the liver of some animals treated with the drug. These pathological effects did not appear to alter the diabetic picture to any appreciable extent.

In the present studies, one series of rats with severe alloxan diabetes was observed for a period of 28 days to check the permanence of the diabetes. Another series with severe diabetes was studied for a preliminary period of 10 days prior to bilateral adrenalectomy. Chemical studies were continued on these rats postoperatively for 9 days.

In both series female rats of the Long-Evans strain (200–230 gm.) were given intraperitoneal injections of 150 mg./kg. of alloxan monohydrate. With this dosage, from 60–70% of the rats developed a severe diabetes, a few had a transitory diabetes and the rest failed to respond to the drug. When a larger dose of alloxan was used, a larger percentage of the animals became diabetic. However, with such doses the diabetes frequently became so severe (blood sugar levels above 500 mg.% accompanied by a marked acetonuria) that the animals succumbed to the disease within a few days.

Eleven diabetic rats were used as control animals in the first series, one animal dying during the experiment. Blood and urine studies were made at intervals of 3 or 4 days, but only the data for the 13th and 28th days are included in the tables. The results of the chemical studies on these animals (table 1) indicate that the diabetes was persistent during the period of study with perhaps some increase in severity. This is shown by the slight increase in the levels of blood and urine glucose at the 28th day period.

In order to test the effect of adrenalectomy on alloxan diabetes, another series of 11 rats was studied. As in the case of the first series, blood and urine studies were made at intervals in order to ascertain the severity of the diabetes before the animals were adrenalectomized. Three of the animals died following adrenalectomy and one other was moribund. The control data for these 4 animals are not included in the tabulation. The blood and urine glucose studies for the 7 remaining rats, 10 days following the injection of alloxan, is given in table 2. All of the rats had a marked diabetes, but the

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TABLE 1. BLOOD AND URINE GLUCOSE IN 10 CONTROL RATS WITH SEVERE ALLOXAN DIABETES

Blood glucose[1] Mg. % Avg.	Highest	Urine vol. cc./24 hrs. Avg.	Highest	Urine glucose gm./24 hrs. Avg.	Highest	Acetone bodies
(a) 13 days following injection of alloxan						
412	495	108	140	8.58	10.64	2 showed 1+
(b) 28 days following injection of alloxan						
423	486	112	139	9.54	11.20	3 showed 1–2+

[1] Non-fasting levels.

disease was not quite as severe as in the animals of the first series studied (see table 1).

The seven rats were adrenalectomized 10 days following the injection of alloxan. Immediately following the operation each animal was given a single injection of 0.5 cc. of Upjohn's adrenal cortical extract.[1] Thereafter, adrenal cortical extract (0.25 cc. daily) was given only to rats whose carbohydrate levels approached the normal non-fasting range. In general, the well-being of the rats which withstood adrenalectomy was improved after the operation. The blood and urine glucose values for these rats, 19 days following the injection of alloxan but 9 days following adrenalectomy, are shown in table 2.

TABLE 2. BLOOD AND URINE GLUCOSE IN 7 RATS WITH ALLOXAN DIABETES BEFORE AND AFTER ADRENALECTOMY

Blood glucose[1] Mg. % Avg.	Highest	Urine vol. cc./24 hrs. Avg.	Highest	Urine glucose gm./24 hrs. Avg.	Highest	Acetone bodies
(a) 10 days following injection of alloxan but before adrenalectomy						
360	404	77	123	5.88	10.39	3 showed 1–3+
(b) 19 days following injection of alloxan but 9 days following adrenalectomy						
126	200	20	24	0.17	0.46	None

[1] Non-fasting levels.

In each of the 7 rats, adrenalectomy caused a marked reduction or complete disappearance of their diabetic symptoms. Important in this regard is the absence of acetone bodies in the urine of these animals. These data on alloxan diabetes are in agreement with observations of Long and Lukens (1936) and Long, Fry and Thompson (1938) who adrenalectomized animals which previously had been pancreatectomized.

Conclusions.—Rats of the Long-Evans strain become severely diabetic following the administration of alloxan. After adrenalectomy, however, there is a marked reduction or a complete disappearance of diabetic symptoms. Thus, animals with alloxan diabetes respond to adrenalectomy in a manner similar to that shown by pancreatectomized animals.

R. G. JANES[2] AND C. E. FRIEDGOOD

From the Department of Anatomy,
Wayne University College of Medicine,
Detroit, Michigan

[1] Supplied through the kindness of Dr. F. G. Cartland of the Upjohn Company.

[2] Present address—Department of Anatomy, State University of Iowa, Iowa City, Iowa.

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EFFECT OF GRADED DOSES OF THYROXINE ON METABOLISM AND THYROID WEIGHT OF RATS TREATED WITH THIOURACIL

UNTIL QUITE RECENTLY investigations of thyroidal activity and the assay of thyroid substance in mammals have depended very largely on determinations of the metabolic rate. Studies of normal thyroid physiology have been been restricted because of the time involved in determinations of metabolism as well as the quantitative limitations.

Since discovery of the goitrogenic action of sulfaguanidine (Mackenzie, Mackenzie and McCollum, 1943) allyl thiourea (Kennedy, 1942), and phenylthiourea (Richter and Clisby, 1942) it has been shown that a large number of the sulfonamides, thioureas and certain aniline derivatives will produce this effect (Mackenzie and Mackenzie, 1943; Astwood, 1943). It appears well established from these latter studies that the enlargement of the thyroid gland is due to inhibition of thyroid hormone formation, which in turn permits increased secretion of thyrotropic hormone by the pituitary and compensatory hypertrophy of the thyroid gland. Thyroid enlargement can be prevented by administering thyroxine or thyroid hormone in sufficient amounts to bring the pituitary and circulating thyroid hormone into normal balance. Thus a new technique is made available for studies of thyroid physiology.

The opposing effects of thiouracil or thiourea and thyroxine were first used by Dempsey and Astwood (1943) to estimate the rate of thyroid secretion in rats at various temperatures and by Mixner, *et al.* (1944) for the assay of thyroidal activity in chicks. It has been shown by Mackenzie and Mackenzie (1943) and Astwood, *et al.* (1943) that the metabolic rate is depressed by these drugs, and also that animals so treated will still respond to thyroxine stimulation. Insufficient data were presented, however, to determine how closely the rate of increase in metabolic rate and the decline in thyroid size of animals given thiouracil plus graded doses of thyroxine will correspond.

In view of the broad possibilities for use of this new technique it seemed desirable to establish more fully the quantitative relation between the increase in metabolic rate and decrease in thyroid gland weight of such animals.

MATERIALS AND METHODS

Groups of young male albino rats fed on Purina dog chow were given 0.1 per cent thiouracil in their drinking water for 14 days. Crystalline, d,l-thy-

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roxine dissolved in distilled water with the aid of the minimum amount of N/10 sodium hydroxide, was injected once daily in amounts varying from 1.25 to 7.50 micrograms daily throughout the experimental period. One group of rats receiving thiouracil and one group of normal rats were retained as controls. The animal room temperature was maintained at 25° to 27° C.

The metabolic rate was estimated by determining the carbon dioxide output of individual rats in a modified Haldane, open circuit respiration apparatus at a temperature of 29° C. on the day before thiouracil administration began and on alternate days, so far as possible, thereafter. After 14 days on experiment the rats were killed with ether and the thyroids were removed, dissected free of fat and connective tissue and weighed on a precision balance to the nearest 0.1 mg. Both metabolism and thyroid weight data were obtained on dosage levels of 0, 1.25, 2.50 and 5.00 micrograms of d,l-thyroxine daily. Thyroid weights alone were obtained from additional groups receiving 3.75 and 7.5 micrograms of d,l-thyroxine and additional thyroid data are also included with certain of the metabolism groups. The normal carbon dioxide output was established by averaging the results of the preliminary day's values for all of the dosage groups together with 6 sets of determinations on the normal control rats obtained during the experimental period. To obtain the final carbon dioxide output of the experimental rats the results of 3 sets of determinations on each group, obtained between the 8th and 14th day of the experiment were averaged.

RESULTS

The normal rats (table 1) had a metabolic rate equivalent to the output of 286.4 mg. of carbon dioxide per 100 gm. body weight per hour and a thyroid

TABLE 1. THE EFFECT OF THIOURACIL ALONE AND THIOURACIL PLUS GRADED DOSES OF THYROXINE ON METABOLISM AND THYROID WEIGHT OF IMMATURE MALE RATS

Metabolism Data						Thyroid Weight Data				
Group No.	Thyroxine daily per rat	No. of rats	Ave. CO_2 output per 100 gm. rat	No. CO_2 det'ns.	Stand. Dev.	No. of rats	Ave. body wt. when killed	Ave. thyroid wt. per 100 gm. rat	Stand. Dev.	Remarks
	(γ)		(mg.)				(gm.)	(mg.)		
1	0	30	286.4	48	30.21	9	133.8	8.97	0.9962	Normal Controls
2	0	8	218.4	21	29.13	8	142.1	19.60	4.1060	0.1% thiouracil in drinking water
3	1.25	4	242.9	12	22.45	8	145.5	15.10	1.7762	0.1% thiouracil in drinking water
4	2.50	8	256.9	21	28.58	12	137.6	12.75	1.4496	0.1% thiouracil in drinking water
5	3.75					7	119.5	11.40	2.0307	0.1% thiouracil in drinking water
6	5.00	8	290.0	20	30.06	17	140.4	8.64	1.8899	0.1% thiouracil in drinking water
7	7.50					8	146.8	8.45	1.8067	0.1% thiouracil in drinking water

weight of 8.97 mg. per 100 gm. body weight. Administration of 0.1% thiouracil in the drinking water (group 2) depressed the carbon dioxide output to 218.4 mg. per 100 gm. body weight per hour and permitted enlargement of the thyroid to 19.6 mg. per 100 gm. body weight. Thus the metabolic rate decreased 23.7 per cent and the thyroid more than doubled in weight as a result of the 2-week treatment with thiouracil.

The reciprocal relationship between the metabolic rate and thyroid gland weight in rats receiving thiouracil alone and thiouracil plus various doses of thyroxine is demonstrated in figure 1. With increasing amounts of thyroxine

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the carbon dioxide output increased in proportion to dosage, reaching the normal, when approximately 4.75 micrograms of d,1-thyroxine was injected daily. The thyroid weight was depressed to the normal level by a daily dosage of approximately 4.8 micrograms of d,1-thyroxine daily.

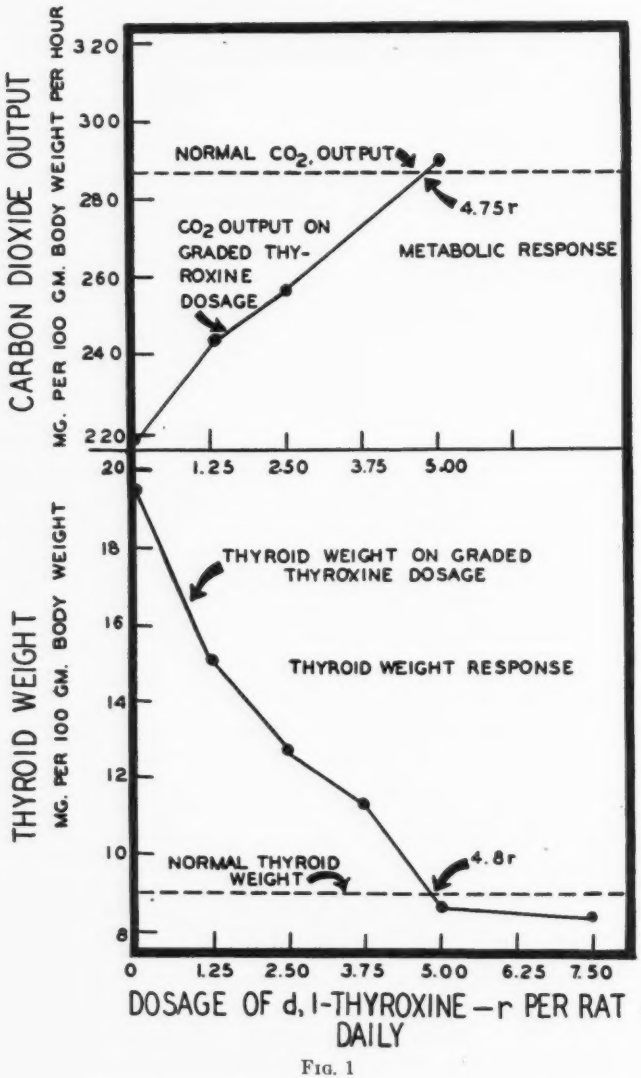

FIG. 1

Since this amount of thyroxine was required to keep the thyroid and the pituitary in balance in the absence of natural thyroid secretion it can be assumed to represent the rate of thyroid hormone secretion of young normal

rats kept under the conditions of the experiments. As shown by the standard deviations (table 1) there is a sizable experimental error involved in measurements of this type, particularly in the determinations of carbon dioxide output. However, the thyroid hormone secretion as measured by the two methods was practically identical. Therefore, it is believed that the results of thyroid assays or measurements of thyroid function obtained by the new thiouracil technique are directly comparable with results of the standard metabolic methods.

SUMMARY

Young male albino rats were given one-tenth per cent thiouracil in their drinking water for a period of 2 weeks in order to block the formation of thyroid hormone. The effect of thiouracil alone and thiouracil plus graded doses of thyroxine on the metabolic rate and the thyroid gland weight was determined. The metabolic rate was depressed 23.7 per cent and the thyroid weight was more than doubled in the rats receiving thiouracil alone. The metabolic rate was returned to normal by daily injection of 4.75 micrograms of d,1-thyroxine. Approximately 4.8 micrograms of d,1-thyroxine was required to return the thyroid weight to normal. From these results it is believed that thyroid assays or measurements of thyroid function determined by the new thiouracil technique are directly comparable with results obtained by the standard metabolic method.

E. P. Reineke, John P. Mixner[1] and C. W. Turner
From the Department of Dairy Husbandry,
University of Missouri,
Columbia, Missouri

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[1] Now Instructor of Zoology, Louisiana State University, Baton Rouge, Louisiana.
Contribution from the Department of Dairy Husbandry, Missouri Agricultural Experiment Station, Journal Series No. 957.

ANTIHORMONE FORMATION FOLLOWING THE ADMINISTRATION OF GONADOTROPHIC EXTRACTS

It is well known that antigonadotrophins form in the blood of experimental animals following the administration of pregnant mare serum. Furthermore antagonists to pregnant mare serum develop in the human and consequently present a complicated factor in the clinical use of this hormone

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(Rowlands and Spence, 1939; Jailer and Leathem, 1940; Leathem and Abarbanel, 1943). Recently another preparation involving a combination of sheep anterior pituitary extract and human chorionic gonadotrophin (Synapoidin) has been made available for clinical use. Mazer and Ravetz (1941) reported ovarian stimulation in 20 of 23 patients treated with this combination of gonadotrophins and since that time various clinical results have been obtained. Limited tests for the presence of antigonadotrophins in the sera of patients following treatment with this extract have yielded negative results (Jailer and Leathem, 1940; Leathem and Abarbanel). On the other hand, allergic manifestations have been noted in patients treated with Synapoidin (Phillips, 1942; Davis, Madden and Hamblen, 1943) but this does not preclude the formation of antigonadotrophins. To establish the validity of test methods for the detection of antigonadotrophins to the combination of gonadotrophins it was deemed of interest to investigate the antigenicity of this material in rabbits. Data on the mode of administration as an influence on the development of antigonadotrophins and the comparative reaction of immature rats and mice to the antisera are presented.

MATERIALS AND METHODS

For these experiments, eight male rabbits weighing approximately 3 kilograms were used. Each animal was injected once daily with 4.5 R.U. (0.3 cc.) of the combination of gonadotrophins (Synapoidin[1]) either subcutaneously or intravenously. The hormone was administered on the first four days of each week for 3 successive weeks. A 10 cc. blood sample was drawn from the heart 6 to 9 days after the last injection. Following a 17 to 25-day interval after the last injection, a second series of injections was administered in the manner indicated but extended over only a 2-week period. The animals were bled to death 7 days after the last injection.

To test for antigonadotrophins, 22-day-old female rats were used. All test rats received a total of 3 R.U. of the hormone and, at a different site, 0.9 cc. of serum. Injections were made subcutaneouly once daily for 3 days and the animals killed 24 hours after the last injection at which time the weights of the ovaries and uteri were obtained, the latter after removal of intra-uterine fluid. The response of litter-mate rats was compared and at least 4 rats were used for each serum test. The antigonadotrophic reaction was graded as negative, partial or complete as determined by the degree to which the serum inhibited the ovarian weight increase anticipated with the gonadotrophin alone. The sera were kept in a refrigerator and tests were started within 48 hours after the blood samples were taken.

RESULTS AND DISCUSSION

Following the first injection series, when the hormones were administered subcutaneously, the sera were found to be completely inhibitory in 2 cases, partial in one case and negative in one case. After the second series of injections, the sera of all rabbits completely inhibited the gonadotrophic activity of 3 R.U. of Synapoidin. Data obtained from one rabbit (#9) are shown in table 1. It should be noted that as little as 0.3 cc. of serum was equally as antagonistic as 0.9 cc. serum.

[1] Synapoidin was generously supplied by Dr. D. K. Kitchen of Parke, Davis and Company.

Four rabbits received the gonadotrophic extracts intravenously and following the first injection series all tests for antigonadotrophins were negative. In each case the rabbits were subjected to a second injection series and a partially inhibitory serum was developed in 2 rabbits (table 1) whereas the other 2 rabbits remained negative. This is in marked contrast to the results obtained when the subcutaneous route of administration is used and may be partially explained on the basis of a more rapid excretion of the gonadotrophins by the kidney when injected directly into circulation. Therefore

TABLE 1. ACTION OF ANTIGONADOTROPHIC RABBIT SERA

No. of rats	Treatment Synap. R.U.	Treatment Serum cc.	Body weight gms.	Avg. ovarian wt. mgs.	Ave. uterine wt. mgs.
		Controls			
15	none	none	38	15.8	28
		Subcu. injections-series #1 Rabbit #9			
4	3	0.9	41	17.8	44
3	3	none	42	29.0	79
		Subcu. injections-series #2 Rabbit #9			
2	3	0.9	57	15.3	29
2	3	0.3	40	18.2	36
4	3	none	48	42.5	85
		Intrav. injections-series #1 Rabbit #8			
2	3	0.9	50	41.7	67
2	3	none	47	37.4	82
		Intrav. injections-series #2 Rabbit #8			
2	3	0.9	43	30.0	82
2	3	none	44	50.0	69

altering the route of administration to lower the tendency toward antihormone formation also lowers the gonadotrophic stimulating potency of the hormone. That the route of administration does influence the ovarian response to the combination of anterior pituitary extract and chorionic gonadotrophin is shown by comparing the subcutaneous and intraperitoneal routes of administration in 22-day-old rats. One finds a 3–4 fold increase in ovarian weight in 72 hours when the 3 R.U. dose is injected subcutaneously whereas the intraperitoneal route results in questionable ovarian weight increase and infrequent uterine stimulation. Whereas these data favor the subcutaneous route of administration for the development of antigonadotrophins Lerman (1942) favors the intraperitoneal route of injection to demonstrate the antigenicity of thyroglobulin.

In previous investigations on the antigenicity of equine gonadotrophin the mouse was used as the test animal. For comparison, several of the sera from Synapoidin treated rabbits that were known to be inhibitory in rats were retested in 22-day-old female mice by the same procedure. In one test the ovaries of 4 control mice receiving 3 R.U. of the gonadotrophins averaged 5.1 mg. whereas the ovaries of 3 mice that were simultaneously injected with

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sera averaged 2.4 mg., the identical weight of uninjected controls. Uterine weight increase too was inhibited. Therefore the mouse or rat can serve as a test animal for these antigonadotrophins. Furthermore the male mouse can also serve as a test animal by injecting 22-day-old mice over a 5-day period with 3 R.U. of the hormone. Seminal vesicle weight increased to 23.3 mg. in mice under this treatment as compared with 6.7 mg. in the controls. If an inhibitory serum was injected with the hormone, no stimulating action was evident, the seminal vesicles being 5.5 mg. in weight.

It is evident that the combination of sheep anterior pituitary extract plus chorionic gonadotrophin will cause the formation of antigonadotrophins in the blood of rabbits in much the same manner as a crude whole sheep pituitary extract (Meyer and Kupperman, 1939) and while this does not preclude a similar action in man it is evident that the possibility exists since the protein being administered is foreign to rabbit and to man.

SUMMARY

A combination of sheep anterior pituitary extract and human chorionic gonadotrophin (Synapoidin) elicits the formation of antigonadotrophins in the rabbit. These antagonists nullify the gonad stimulating action of the hormones in female rats and in female and male mice. Subcutaneous injections will cause antigonadotrophin formation more readily than intravenous injections.

J. H. LEATHEM

From the Department of Zoology,
Rutgers University,
New Brunswick, New Jersey

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THE ALLEGED ENDOCRINE EFFECT OF YOHIMBINE[1,2]

Different attempts to demonstrate the pharmacological action of yohimbine on sex organs with an adequate experimental method have not produced consistent results. Rumry (1918) found no change of reproductive rate in adult mice given injections of yohimbine. B. Zondek (1926) found yohimbine to be without effect in the female castrated mouse (Allen-Doisy Test). Klein

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[1] Generously aided by a grant from the Ella Sachs Plotz Foundation.
[2] Because of the difficulties of international communication the authors have not read proof of this article.

(1927) was unable with yohimbine to provoke estrus and to prevent uterine involution following spaying in rabbits. Similarly Loewe, Lange, Voss and Pass (1927) and D'Amour (1935) found yohimbine without action on the vagina of infantile and castrated rats. On the other hand, Ludwig and von Ries (1931) and Hechter, Lev and Soskin (1940) noted positive estrogenic effects with yohimbine.

Fugo and Gross (1942) found yohimbine without effect on the sexual organs of infantile female rats, but noted in adult female rats permanent estrus in response to small doses (1–2 mg. per day), and pseudopregnancy in response to larger doses (3–4 mg. per day) of yohimbine. Castrated or hypophysectomized rats did not react. The authors concluded that yohimbine stimulates the release of luteinizing hormone by the pituitary gland. They also found atypical sexual behavior during periods of heat in yohimbine treated rats. Hamblen (1943) in an editorial of the *Journal of Clinical Endocrinology* has suggested that yohimbine, if its alleged estrogenic effect is in fact mediated by a stimulating influence on the pituitary gland, may prove of general endocrinological value.

We have studied the effect of yohimbine on 120 infantile female and male rats, 35 adult female rats, and 20 adult spayed female mice. Following subcutaneous injections of yohimbine hydrochloride the following findings were noted.

1) No estrogenic effect with 0.3–3 mg. per day yohimbine HCl in Allen-Doisy Tests with 10 adult spayed female mice and 12 infantile female rats. Higher doses of yohimbine were toxic.

2) No augmentation of the estrogenic effect of threshold doses of estrone[3] with 3 mg. per day yohimbine HCl in 10 adult spayed female mice.

3) No acceleration in the appearance of the first estrus in 20 infantile female rats injected with $\frac{1}{2}$–2 mg. yohimbine HCl daily from the 21st to the 50th day of life, estrus appearing on the 50th day of life ($\pm$ 4 days) both in the yohimbine treated group and in the controls.

4) No gonadotropic effect in 12 infantile female and 12 infantile male rats with 0.5–5 mg. per day yohimbine HCl given by injection for 3 days.

5) No augmentation of response to a threshold dose of chorionic gonadotropin[4] in 20 female infantile female rats injected with 0.5 mg. yohimbine HCl daily.

6) No induced difference in the time of appearance and in the periodicity of vaginal estrus and formation of corpora lutea in 24 female rats 3-weeks-old treated to an age of 5 months with daily doses of 0.1–3 mg. yohimbine HCl.

7) No hyperemia in the sexual organs of 24 3-weeks-old male rats treated to an age of 5 months with daily doses of 0.5–3 mg. yohimbine HCl. No difference in the size and weight of sex organs after 3 months between treated males and controls. In both groups descent of the testes occurred between the 45th and 50th day of life. Nose bleeding was encountered occasionally.

8) No changes in the appearance of the vaginal smear in twenty 60–100 day old female rats treated with daily injections of 1–4 mg. yohimbine HCl for 5 weeks. No difference was induced in body-weight, weight of ovaries, and

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[3] We are indebted to Dr. B. J. Brent (Roche-Organon, Nutley N. J.) for the supply of estrone.

[4] We are indebted to the Winthrop Chemical Co., N. Y., for the supply of Korotrin.

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in the number of corpora lutea. A group of animals treated with larger doses of yohimbine HCl (3–4 mg. daily) died on an exceptionally hot and dry day.

In the series Nos. 6–8 of our experiments we noted excitement and irritability in rats chronically treated with high doses of yohimbine. The sexual organs of the males and females of these test groups showed no special hyperemia, nor were there spontaneous erections of the penis. There was no evidence of pituitary stimulation, the body, pituitary and ovary weights and the endocrine reactions of the animals being normal.

In consideration of the results presented above and the negative impression gained from clinical data appears an unbridgeable gap between earlier positive reports on the endocrinological activity of yohimbine and our own negative results. We have tested 5 different preparations of yohimbine hydrochloride: British Drug House, Boehringer, Gehe, Riedel and a local preparation. Experiments with other yohimbine brands seem desirable before a final decision concerning the alleged pharmacological effect of this drug on the sexual organs is reached.

We wish to thank Prof. B. Zondek under whose supervision this work was done.

F. Sulman
R. Black

Hormone Research Laboratory,
Hebrew University, Jerusalem, Palestine

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THE ANTITHYROID ACTIVITY OF THIOBARBITAL (5,5-DIETHYL-2-THIOBARBITURIC ACID)[1]

Among a group of 103 compounds tested for their antithyroid effects in rats, thiobarbituric acid was reported to be second only to thiouracil in its activity (Astwood, 1943). The high potency and the relatively low toxicity prompted a further investigation of this compound preliminary to a trial of its effectiveness in human beings suffering from hyperthyroidism.

It was soon apparent that an error had been made and that thiobarbituric acid was practically inert. Fortunately a sample of the original compound labeled thiobarbituric acid was still available. Repeated tests in rats con-

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firmed the original estimate of potency and it was clear that this substance was not thiobarbituric acid. The active compound was found to melt sharply at 175–176°C. (uncorrected), to be almost tasteless and to have the elementary composition: C = 47.73, 47.61%; H = 5.62, 5.60%; N = 14.46, 14.60%; S = 16.14, 16.22%. Calculations for $C_8H_{12}N_2SO_2$ gave: C = 47.98%; H = 6.04%; N = 13.99%; S = 16.10%. It seemed probable therefore that the

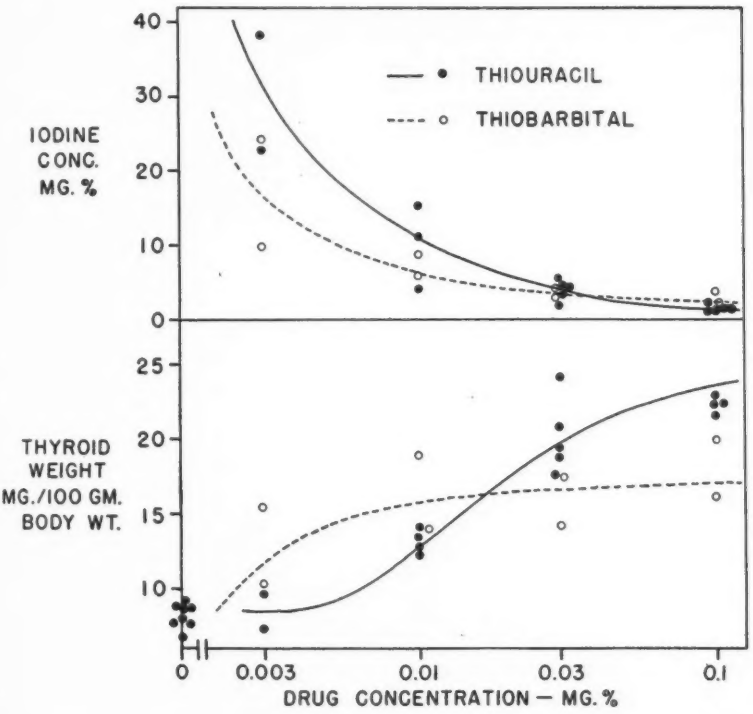

FIGURE 1. Effect of thiouracil and of thiobarbital when fed to rats for 10 days at various concentrations in the diet (logarithmic scale) on the iodine concentration and weight of the thyroid gland.

active material was the diethyl derivative. Two authentic samples of 5,5-diethyl-2-thiobarbituric acid[2] were found to melt at 175–176°C., and when mixed with the original compound there was no depression of the melting point. Furthermore, tests in rats showed that 5,5-diethyl-2-thiobarbituric acid and the original compound were of the same activity.

Thus, there appears to be no doubt that the compound originally reported as thiobarbituric acid was indeed thiobarbital; the similarity of the common names was probably responsible for the error.

Assayed in rats, using a method recently published (Astwood and Bissell,

[2] Kindly supplied by the Abbott Laboratories, North Chicago, Illinois and by the Lederle Laboratories, Pearl River, New York.

1944), thiobarbital gave the results shown in figure 1. It was found that in the lower dosages thiobarbital was more active than thiouracil, while in the higher levels of dosage it appeared to be less active; as the dosage-response relationships differed it was not possible from these data to state accurately the relative activities of the two compounds. However, it would appear that in the dosages which correspond most closely with those used in man thiobarbital is distinctly more active than thiouracil. The dosage of thiouracil used in human beings is usually less than 0.5 Gm. per day—or for a 70 Kg. man less than 7 mg. per Kg. of body weight per day. The concentrations 0.003 and 0.01% of the ration provided an average daily intake of roughly 5 and 16 mg. per Kg. of body weight per day respectively. Comparing the dosage response curves of figure 1 in this region indicates that thiobarbital is 1.5 to 3 times as active as thiouracil in this test.

The relative toxicity of the two compounds was also difficult to estimate. The rate of growth in the 10 day feeding experiments showed thiobarbital to be distinctly less toxic than thiouracil when administered in the food at all concentrations up to 0.3%. When given in a concentration of 1% of the ration, thiouracil was tolerated by most of the animals for many weeks, while thiobarbital at this concentration induced a state of marked depression with hyperirritability; these animals lost weight and died.

Two litters of rats were treated from the 18th day of life for a period of eight months with 0.1% solutions of the two drugs in the drinking water. The growth rate ran parallel in the two groups for the first 5 months; the usual plateau of hypothyroidism occurred at the same time (3 months) at body weights averaging 135 grams. During the last 3 months the animals receiving thiobarbital solution continued to gain slowly while those receiving thiouracil did not. During the first 5 months the volume of solution consumed daily by each rat averaged 22.5 cc. of thiobarbital solution and 16.1 cc. of thiouracil solution. This difference in fluid intake persisted to the end of the experiment, but its cause was not determined. At autopsy at the end of the 8 months of treatment the thyroid glands averaged 87 and 93 mg. per 100 Gm. body weight for the rats receiving thiouracil and thiobarbital respectively; the only significant pathological change observed was a fatty infiltration of the livers of the animals receiving thiobarbital, similar to that described by Richards and Appel (1941) in dogs given thiobarbituric acid derivatives.

Tests on 2-thiobarbituric acid showed that concentrations as high as 1% of the ration resulted in no thyroid enlargement in 10 days and only a questionably significant reduction in the iodine concentration of the thyroid from an average of 70.4 mg.% in the controls of 49.2 mg.% in the animals given the 1% concentration in the diet.

Summary. These experiments indicate that in rats thiobarbital in small doses is somewhat more effective in inhibiting thyroid function and is possibly less toxic than thiouracil.

E. B. Astwood, Adele Bissell and A. M. Hughes
From the Departments of Medicine and Pharmacology, Harvard Medical School.

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NEW BOOKS

OFFICE ENDOCRINOLOGY. By Robert B. Greenblatt, B.A., M.D., C.M. Professor of Experimental Medicine, University of Georgia School of Medicine; Director, Sex Endocrine Clinic University Hospital, Augusta, Georgia. xii, 243 pages, 48 figures, price: $4.00. Published by Charles C Thomas, Springfield, Illinois.

This book is designed as a handbook for general practitioners of medicine. Emphasis is given chiefly to the diseases of the gonads. Very few pages are allocated to the thyroid or to the adrenal glands. The fundamental physiology of the sex glands is reviewed briefly and in an acceptable fashion.

From the point of view of diagnosis the advice offered is, on the whole, sound. Many of the modern diagnostic procedures are described and discussed. However, such useful indices of endocrine function as the urinary ketosteroids and the urinary follicle stimulating hormone are hardly mentioned. Throughout the sections dealing with diagnosis there is a tendency to lean towards an endocrine diagnosis. Possibly this tendency may be misleading at times.

Of more concern to the reviewer is the great enthusiasm of the author for endocrine therapy. Thyroid, estrogens, androgens, adrenal cortical preparations, etc., are recommended for many conditions. It is believed that a more critical judgment of the clear indications for specific endocrine therapy would add to the value of this book. A short but reasonably good bibliography is appended to each chapter.

NATHAN B. TALBOT

In the review of Dr. Max A. Goldzieher's book "The Adrenal Glands in Health and Disease" which appeared in the November issue of ENDOCRINOLOGY, the price was incorrectly listed as $18.00. The correct price of this book is $8.00. (Editor's Note.)

ASSOCIATION NOTICE

ANNOUNCEMENT OF ANNUAL MEETING

The Twenty-Eighth Annual Meeting of The Association for the Study of Internal Secretions will be held in New York City on Monday and Tuesday, June 18 and 19, 1945. Headquarters will be at the Commodore Hotel. The Chairman of the Local Committee is Dr. Earl T. Engle, 630 West 168th Street, New York City assisted by Dr. Robert Gaunt, New York University, who will be in charge of projection arrangements.

Those wishing to present papers should send the title of the paper and four copies of a comprehensive abstract to the President, Dr. Carl R. Moore, Department of Zoology, The University of Chicago, Chicago 37, Illinois, not later than March 15, 1945.

CARL R. MOORE, *President*
HENRY H. TURNER, *Secretary*

ASSOCIATION ANNOUNCEMENT

Nominations for Awards of the Association

Two awards for meritorious work in endocrinology will be given at the next annual meeting of the Association. The work may be either clinical or in the basic sciences. A special Committee of five members of the Association chooses the recipients of these Awards, subject to ratification by the Council.

Each member of the Association has the privilege of making one nomination for each Award. A nomination should be accompanied by a statement of the importance of the nominee's contributions in endocrinology and by a bibliography of his most important papers with reprints if possible. Five copies should be sent to the Secretary, Dr. Henry H. Turner, 1200 North Walker Street, Oklahoma City, Oklahoma, *not later than March 15, 1945*. The Awards are described below.

THE E. R. SQUIBB AND SONS AWARD

The E. R. Squibb and Sons Award of $1,000.00 was established in 1939. It was given in 1940 to Dr. George W. Corner, in 1941 to Dr. Philip E. Smith, in 1942 to Dr. Fred C. Koch, and in 1944 to Dr. Edward A. Doisy. No age or other special limitation is stipulated by the Donor of the Award. So far it has been given for long-continued work of a high quality.

THE CIBA AWARD

The Ciba Award was established in 1942 and is to be given to an investigator not over 35 years of age. No recipient was selected in 1942. In 1944 the Award was given to Dr. E. B. Astwood. The Award is for $1,200.00. If the recipient chooses to use the Award to aid in working in a laboratory other than the one in which he normally is located, the Award will be increased to $1,800.00.

CARL R. MOORE, *President*
HENRY H. TURNER, *Secretary*

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